INVESTIGATING THE FUNCTION AND THERAPEUTIC POTENTIAL OF THE GCN5B BROMODOMAIN IN *TOXOPLASMA GONDII*

Jocelyne Nicole Hanquier

Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Master of Science
in the Department of Microbiology and Immunology,
Indiana University

June 2020

Accepted by the Graduate Faculty of Indiana University, in partial fulfillment of the requirements for the degree of Master of Science.

Master's Thesis Committee

_____
William J. Sullivan, PhD, Chair

_____
Gustavo Arrizabalaga, PhD

_____
Stacey Gilk, PhD

DEDICATION

To Carol, with love.

“Tomorrow belongs to those who can hear it coming.”-David Bowie

ACKNOWLEDGEMENTS

I would like to express my most sincere gratitude to the mentors, teachers, and friends who have helped me on my scientific journey thus far. Firstly, I would like to thank my mentor Dr. William J. Sullivan for the opportunity to work in the Sullivan lab, for teaching me how to think critically about science, and for establishing a lab with amazing post-docs and students from which to learn. I would also like to thank the other members of my thesis committee, Drs. Gustavo Arrizabalaga and Stacey Gilk, for the integral feedback, guidance, and discussions that helped drive the project forward.

I extend a special thanks to Victoria Jeffers, who acted as my closest mentor. Thank you for your generosity, patience, and wisdom. You made all of the difference in my experience. And to the labmates with whom I worked most closely: Leonardo Augusto, Michael Holmes, Jennifer Martynowicz, and Sandeep Srivastava- thank you all for your guidance and friendship. Thomas Gimeno, thank you for your experimental help with the binding assays, as well as making summers in the lab fun and exciting. I would also like to acknowledge the other current and past members of the Sullivan lab.

I would like to thank Alyssa Ruble and Colin Hailey for being part of my extended family, and my girlfriend, Catherine Wheeler, for being my best friend and for believing in me. Lastly, I would like to thank my family: Carol, Jose, Zachary, Zoe, and Gabriella Hanquier, for the unconditional love and continuous support throughout my life and academic career-you give me the strength to persist.

Jocelyne Nicole Hanquier

INVESTIGATING THE FUNCTION AND THERAPEUTIC POTENTIAL OF THE GCN5B BROMODOMAIN IN *TOXOPLASMA GONDII*

The obligate intracellular protozoan parasite *Toxoplasma gondii* is a medically relevant pathogen that has infected a third of the world's population. *Toxoplasma* is the causative agent of toxoplasmosis, which can have severe repercussions such as encephalitis and even death in immunocompromised patients. Current treatments for toxoplasmosis only target acute infection and can be toxic to patients, resulting in complications including allergy and bone marrow suppression. Thus, the identification of novel drug targets and therapies for toxoplasmosis is vital. Epigenetic modulators of lysine acetylation, including 'writers,' 'erasers,' and 'readers,' have been identified as promising drug targets for protozoan parasites. The lysine acetyltransferase (KAT) GCN5b appears to be an essential gene for *Toxoplasma* viability. The KAT domain of GCN5b is essential to GCN5b function and is targetable by small molecule inhibitors. While the acetyltransferase activity of this gene is well-characterized, the functionality of its C-terminal bromodomain (BRD) remains to be understood. Bromodomains are readers of lysine acetylation, and recently, bromodomain inhibitors have shown promise in a number of human diseases, as well as in protozoan parasites. We hypothesized that the GCN5b bromodomain is critical for *Toxoplasma* viability. The data reported herein suggest that the GCN5b

bromodomain is important for tachyzoite viability and may serve as a novel therapeutic target in *Toxoplasma*.

William J. Sullivan, PhD, Chair

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Ac	acetyl
ADA2	Alteration/deficiency in activation protein-2
AID	auxin inducible degron
ANOVA	analysis of variance
AP2	Apetala-2
ATPase	enzyme that catalyzes hydrolysis of adenosine triphosphate
BAZ2A	Bromodomain adjacent to zinc finger domain-2A
BC	alpha helix B to alpha helix C
bp	base pair
BRD	bromodomain
BRPF1	Bromodomain and PHD finger-containing protein-1
BSA	bovine serum albumin
CBP	CREB binding protein
CECR2	Cat eye syndrome chromosome region-2
ChIP-chip	chromatin immunoprecipitation coupled to microarray
CPRG	chlorophenolred-β-D-galactopyranoside
DAPI	4′,6-diamidino-2-phenylindole
dd	destabilization domain
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol

E3	ubiquitin-protein isopeptide
EtOH	ethanol
FALZ	Fetal Alzheimer antigen
FBS	fetal bovine serum
GCN5	General control nonderepressible-5
GNAT	GCN5-related N-acetyltransferase
GST	glutathione-S-transferase
ha	hemagglutinin
HEK-293 cells	human embryonic kidney-293 cells
HFF	human foreskin fibroblast
HIV	human immunodeficiency virus
I-BET151	inhibitor of bromodomain and extraterminal-151
I-Tasser	iterative threading assembly refinement
IC_{50}	inhibitory concentration 50%
IFA	immunofluorescence assay
IPTG	isopropyl β -D-1-thiogalactopyranoside
KAP1	KRAB-associated protein 1
KAT	lysine acetyltransferase
K_D	dissociation constant
kDa	kiloDalton
KDAC	lysine deacetyltransferase
HAT	histone acetyltransferase
mM	milliMolar

MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium
MWCO	molecular weight cut off
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
nm	nanometer
NMR	nuclear magnetic resonance
OD_{600}	optical density at 600 nm
OE	over expressor
page	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCAF	p300/CBP-associated factor
PCR	polymerase chain reaction
PDB	Protein Data Bank
PHD	Plant homeodomain
PTM	posttranslational modification
qPCR	quantitative polymerase chain reaction
SAGA	Spt-Ada-GCN5-acetyltransferase
SDS	sodium dodecyl sulfate
SUMO	small ubiquitin-like modifier
Swi/Snf	Switch/Sucrose Non-Fermentable
TAF1/250	TBP-associated factors-1/250
TIF1β	Transcriptional intermediary factor-1β

TRIM33	Tripartite motif-containing 33
v/v	volume for volume
WT	wild-type
ZA	alpha helix Z to alpha helix A
α	alpha
β	beta
ε-N-amino	epsilon nitro amino
µg	microgram
µL	microLiter
µM	microMolar

CHAPTER 1: INTRODUCTION

1.1 *Toxoplasma gondii* is a model apicomplexan parasite

The obligate intracellular protozoan parasite *Toxoplasma gondii* is a model organism for the apicomplexan phylum, which consists of other medically relevant pathogens that infect humans and animals [1]. Some of the apicomplexan parasites that infect humans include *Plasmodium*, the causative agent of malaria, and *Cryptosporidium,* the causative agent of cryptosporidiosis; apicomplexans that infect non-human animals include *Neospora*, which infects cattle, and *Eimeria*, which causes coccidiosis in poultry. The apicomplexans are characterized by a polarized cell structure with a complex arrangement of cytoskeleton and organelles at the apical end [2], including secretory organelles such as rhoptries and micronemes, which are important for parasite adhesion and invasion [3].

The experimental and genetic tractability of *Toxoplasma* make it a useful model organism. *Toxoplasma* is easily propagated in culture and has a well-established mouse model [1]. Furthermore, due to ease in detecting *Toxoplasma* organelles microscopically, *Toxo*plasma has been used to study a number of apicomplexan biological processes including apicoplast development, roles of actin and mysosin in motility and cytoskeleton, and invasion of host cell by secretion of rhoptry contents [1].

Toxoplasma is amenable to genetic manipulation, including stable transient transfection [4], epitope tagging [5], ectopic expression [6], and

generation of reporter genes [7]. *Toxoplasma* is also amenable to genetic disruption by allelic replacement [8] and CRISPR/Cas9 technology [9,10]. In fact, the first genome-wide genetic screening of an apicomplexan was performed in *Toxoplasma*, where CRISPR/Cas9 was utilized to identify essential apicomplexan genes [9]. Additionally, *Toxoplasma* has a fully sequenced genome (12x coverage) and a functional genomic database (ToxoDB) for integrated *Toxoplasma* data, including genomic sequence and annotation, as well as other information such as orthologs, single nucleotide polymorphisms, and proteomics data [11].

1.2 Life cycle of *Toxoplasma*

Toxoplasma has the ability to infect nearly all nucleated cells in warm-blooded animals and has been estimated to have infected about one-third of the world's population [12]. Part of the success in the transmission of *Toxoplasma* is the ability of the parasite to convert between multiple, infectious forms and to infect via different routes [13]. Infectious stages of *Toxoplasma* include: tachyzoites, bradyzoites, and sporozoites, reviewed in [2]. Tachyzoites rapidly proliferate within the intermediate host and non-intestinal epithelial cells of the definitive host, the felid. Tachyzoites actively penetrate through host cells or are phagocytosed. Upon invasion, tachyzoites are surrounded by a parasitophorous vacuole, within which they replicate asexually by endodyogeny (formation of two daughter cells inside a mother cell), and then egress and invade a nearby cell. Tachyzoites can convert into slowly dividing, virtually quiescent bradyzoites that form tissue cysts

throughout the body, predominantly in neural and muscular tissues, including the brain, eyes, skeletal, and cardiac muscles. Bradyzoites characterize the chronic form of *Toxoplasma* infection, as they can persist throughout the life of an individual and are impervious to *Toxoplasma* treatment. Upon immunosuppression, bradyzoites can convert to tachyzoites [14].

The life cycle of *Toxoplasma* is reviewed in [2]. The definitive host of *Toxoplasma*, the cat (Felidae family), is the only organism in which the parasite can undergo the sexual stage of its life cycle. Parasites multiply within the intestinal epithelium of cats, initiating gamete formation. Unsporulated, environmentally stable oocysts (cysts containing *Toxoplasma* zygotes) are shed in the cat feces and are sporulated in the environment within a few days. The sporulated, infectious oocysts can contaminate food, water, and soil and infect intermediate hosts, such as livestock. After ingestion of infectious oocysts, sporozoites develop into tachyzoites, which covert to bradyzoites in tissue cysts. The cat can become infected via consumption of environmental oocysts or tissue cysts from intermediate hosts. Humans can become infected through ingestion of food or water contaminated with oocysts or tissue cysts in infected, undercooked meat. Mothers can vertically transmit *Toxoplasma* to the fetus through the placenta. Additionally, *Toxoplasma* can be acquired through solid organ and hematogenous stem cell transplants, either through reactivation of latent infection or de novo infection due to immunosuppression [15].

1.3 Toxoplasmosis and treatments

Toxoplasma poses a significant health risk to humans and livestock, necessitating the development of effective and safe drugs. Acute infection is characterized by rapidly proliferating tachyzoites but is often asymptomatic in immunocompetent hosts. The healthy immune system controls the infection within weeks, at which point *Toxoplasma* establishes a chronic, latent infection characterized by its conversion into bradyzoite tissue cysts [16]. As the parasite is not eradicated from the host, it can reactivate to cause acute, opportunistic infection in immunocompromised individuals, such as those infected with HIV. Extensive tissue damage can occur during reactivated toxoplasmosis that results in severe complications including ocular toxoplasmosis and *Toxoplasma* encephalitis, which is typically the leading cause of death following reactivation of *Toxoplasma* infection [17]. Additionally, *Toxoplasma* tachyzoites can be transplacentally transmitted from mother to fetus to cause congenital toxoplasmosis, which can result in birth defects, such as neurological and visual impairment and spontaneous abortion [18].

There is currently no treatment against chronic *Toxoplasma* infection (bradyzoite tissue cysts) [13]. The first line of therapy for acute toxoplasmosis is a combination of pyrimethamine and sulfadiazine, often administered with leucovorin to reduce cytotoxicity [19]. Pyrimethamine and sulfadiazine inhibit parasite replication by blocking the folate metabolic pathway, but the treatment is not effective against latent tissue cysts [20]. The treatment is also toxic, putting patients at risk of bone marrow suppression and dermatologic and

gastrointestinal events; discontinuation or change in this treatment regimen due to adverse events occurred in over 55% of studies analyzed in a systematic review [20]. As a result of these shortcomings, there is an urgent need for the identification of novel drug targets to facilitate the development of more effective and better-tolerated treatments for toxoplasmosis.

1.4 Epigenetics and lysine acetylation

A promising area of investigation for potential drug targets in *Toxoplasma* includes protein complexes that “read” and “write” posttranslational modifications (PTMs), including lysine acetylation [21]. The addition and removal of acetyl marks from lysine residues is mediated by lysine acetyltransferases (KATs) and lysine deacetylases (KDACs), respectively. In the literature, enzymes with acetyltransferase activity on lysine residues may also be referred to as ‘histone acetyltransferases’ (HATs), as histones were their initially discovered substrates. However, upon the discovery that acetyltransferases also have non-histones substrates, Allis et al. [22] called for a change in nomenclature to ‘lysine acetyltransferase’ (KAT).

Acetylation of lysine on the ε-N-amino acid neutralizes the positive charge and allows for loosening of chromatin, which is generally linked to transcriptional activation [23]. Lysine acetylation has emerged as a key PTM taking place on hundreds of proteins throughout various compartments in the cell. Extensive lysine acetylation occurs in both *Toxoplasma* and the fellow apicomplexan parasite *Plasmodium* on proteins that function in a variety of areas including

metabolism, translation, chromatin biology, and stress response [24,25]. In addition, lysine acetylation of histones is correlated with stage-specific gene expression and stage conversion in *Toxoplasma* [26].

1.5 GCN5 lysine acetyltransferases

Conservation among organisms:

GCN5 (general control nonderepressible-5) is a nuclear transcriptional co-activator that is highly conserved among vertebrates, plants (*Arabidopsis*), *Drosophila*, yeast, humans, and protozoan parasites [27-29]. GCN5 has conserved functional domains, including a lysine acetyltransferase domain, ADA2-binding domain, and C-terminal bromodomain [27].

GCN5 in yeast and humans:

GCN5 appears to be important for transcriptional activation and stress response. GCN5 has conserved acetyltransferase activity on histone H3 and transcriptional activation of target genes in yeast and humans [30,31]. The acetyltransferase activity of GCN5 is required for the regulation of stress-response genes in evolutionarily divergent yeast species [32]. In *Saccharomyces cerevisiae,* which possess a single GCN5, GCN5 is not essential for growth under normal conditions, but required for growth on minimal media [33]. Humans possess two GCN5 homologues, GCN5L2 (KAT2A) and PCAF (p300/CBP-associated factor) (KAT2B). GCN5 (KAT2A) is required for mouse embryogenesis; while, human PCAF is not [34,35]. Dysregulation of PCAF has

been implicated in disease states including cancer, HIV infection, and neuroinflammation [36]. Both yeast and human GCN5 function as part of ADA2- and SAGA (Spt-Ada-GCN5-acetyltransferase)-containing complexes to drive transcriptional initiation and activation [37].

GCN5 in protozoan parasites:

Plasmodium:

A single GCN5, with a single C-terminal bromodomain, has been identified in *Plasmodium* species. In 2004, Fan et al. [38] identified a GCN5 homologue in *Plasmodium falciparum* with conserved lysine acetyltransferase activity on histone H3 tails. This group found that PfGCN5 associated with PfADA2 *in vitro*, suggesting that GCN5 is involved in chromatin remodeling in *Plasmodium*. Rawat et al. [39] found that GCN5 may serve as a transcriptional regulator of stress responses in *Plasmodium*. They found that PfGCN5 binds to stress responsive genes and, under stress conditions, regulates the expression of genes important for parasite cellular homeostasis. Cui et al. [40] found enrichment of H3K9 acetylation at putative transcriptional initiation sites corresponding to expression of erythrocytic stage-specific genes. Additionally, they found that curcumin, which appears to be an inhibitor of the PfGCN5 acetyltransferase domain [41], led to reduced promoter H3K9 acetylation and gene expression, suggesting that PfGCN5 is recruited to gene promoters to mediate histone acetylation and stage-specific gene expression.

In addition to suggested roles in chromatin remodeling and transcriptional regulation, GCN5 may be important for malarial viability and pathogenicity [39,42]. Treatment of *P. falciparum* with a GCN5-related N-acetyltransferase (GNAT) family KAT inhibitor, embelin, resulted in a reduction in H3K9 acetylation levels around the promoters of var genes, a family of genes important for malarial pathogenicity, and a decrease in var gene expression. Embelin treatment also resulted in inhibition of *Plasmodium* growth at a low micromolar range [42]. A screen of compounds against the PfGCN5 acetyltransferase domain identified molecule C14 as a specific inhibitor of the PfGCN5 acetyltransferase domain. Treatment with C14 reduced recombinant PfGCN5 activity and H3K9 acetylation levels, as well as inhibited *Plasmodium* growth at a low nanomolar range [43]. Additionally, inhibition of GCN5 reverses sensitivity to artemisinin, suggesting that GCN5 may play a role in modulating drug resistance to artemisinins [39]. These data suggest that the GCN5 lysine acetyltransferase domain may serve as a targetable therapeutic domain in *Plasmodium*.

Toxoplasma:

Two GCN5 family KATs have been identified in *Toxoplasma* and designated GCN5a and GCN5b [8]. Each has a lengthy N-terminal extension, a KAT domain, and a single C-terminal bromodomain. The two GCN5's in *Toxoplasma* also show conservation of acetyltransferase activity on histone H3 (though they have different substrate specificities *in vitro*) and formation of ADA2 complexes. GCN5a preferentially acetylates H3K18 [26]; however, GCN5b

acetylates H3K9, 14, and 18 [8]. *Toxoplasma* possesses two ADA2 homologues, ADA2-A and ADA2-B [8]. GCN5a interacts with ADA2-B [8]; however, GCN5b has been associated with at least two distinct protein complexes, both characterized by interaction with ADA2-A [44].

Knockout studies indicate that GCN5a is dispensable for tachyzoite proliferation but required for proper stress responses [45]. Conversely, multiple attempts to knockout GCN5b have failed [45,6]. Wang et al. [6] previously observed that when a catalytically inactive form of GCN5b (GCN5bE703G) was ectopically expressed, it exerted a dominant negative effect, resulting in arrest of parasite proliferation. This supports the idea that GCN5b is essential for tachyzoite viability. Additionally, ChIP-chip analysis demonstrated that GCN5b is enriched at tachyzoite-specific genes involved in transcription, translation, and metabolism [6]. Jeffers et al. [46] demonstrated that the KAT inhibitor garcinol appears to target the KAT domain of GCN5b. Treatment of *Toxoplasma* with garcinol *in vitro* decreases GCN5b auto-acetylation, alters expression of genes linked to GCN5b control, and inhibits parasite replication [46]. These data, along with a recent genome-wide fitness screen reporting a mean phenotype score of -2.96 [9], strongly support GCN5b as an essential gene.

1.6 Bromodomain proteins

Conservation among organisms:

The bromodomain was first identified as a structural motif in the *Drosophila melanogaster* brahma protein, a nuclear chromatin remodeling

protein involved in the activation of homeotic genes [47]. Since then, it has been determined that the bromodomain is a highly conserved domain among humans [48], yeast [49], and protozoan parasites [29] in proteins involved in the regulation of gene expression.

In the human proteome, 61 bromodomains, encoded by 46 proteins, have been identified and structurally clustered into eight major phylogenetic families [48]. The two human GCN5 homologues (GCN5L2 and PCAF) belong to the first phylogenetic family of bromodomains, which also consists of genes FALZ and CECR2. In yeast, 14 bromodomains, encoded by 9 bromodomain-containing proteins, have been identified [49].

In protozoan parasites, bromodomains are conserved among different phylums, including the Euglenozoa phylum (*Trypanosoma cruzi and Trypanosoma brucei)* and the Apicomplexa phylum (*Plasmodium falciparum and Toxoplasma gondii*), reviewed in [29]. There are 12 predicted bromodomain-containing proteins in *Toxoplasma*. Among them, only three have been characterized (histone acetyltransferases TgGCN5a, TgGCN5b, and TAF1/250). The *P. falciparum* GCN5 protein also contains a C-terminal bromodomain.

Structure:

The bromodomain is comprised of ~110 amino acids and folds into a conserved left-handed bundle of four helices (α_Z, α_A, α_B, and α_C) joined by two variable loops (ZA and BC) that form a deep hydrophobic pocket in which the acetyl lysine binds [50]. The amino acid residues within these variable loop

regions determine binding specificity of the acetyl lysine [50]. The interaction between the acetyl lysine and the hydrophobic pocket of the bromodomain is generally stabilized by hydrogen bonding between the acetyl lysine and the ZA and BC loops, specifically hydrogen bonding between the carbonyl group and amide nitrogen in the acetyl lysine and a group of conserved water molecules that hydrogen bond with residues within the ZA loop, as well as hydrogen bonding between the oxygen of the acetyl carbonyl of the acetyl lysine and an amide nitrogen of an asparagine within the BC loop [51].

Binding Activity:

The bromodomain structure was first reported for PCAF by nuclear magnetic resonance (NMR) [50]. NMR titrations of the PCAF bromodomain with a histone H4 peptide containing an acetylated lysine residue demonstrated that the bromodomain binds specifically to acetylated lysine residues [50].

Filippakopoulos et al. [48] assessed the binding specificity of 33 representative human bromodomains for singly acetylated histones *in vitro.* This group observed differences in ligand binding specificities among bromodomains, as well as differences in electrostatic potential surrounding the acetyl-lysine binding sites, suggesting that bromodomains recognize markedly different sequences. Bromodomain binding specificities for singly acetylated histones were generally low; however, in the majority of bromodomains tested, binding specificity for acetylated histones increased in the presence of multiple posttranslational modifications (such as phosphorylation) on histone H3,

suggesting that bromodomain activity is influenced by neighboring modifications.

Function:

Human bromodomain-containing proteins have a variety of functions, reviewed in Table 1 of [52], including roles in: transcriptional initiation (TAF1), regulation (BRD4), elongation (TRIM33), activation (BRPF1), and even repression (BAZ2A), as well as histone acetylation (PCAF), chromatin remodeling (CECR2), and protein scaffolding (BRD1). The majority of these bromodomain-containing proteins localize exclusively to the nucleus, with some also localizing to the cytoplasm [52].

1.7 GCN5 bromodomains

Conservation among organisms:

GCN5 bromodomains have relatively high conservation among species (Table 1) (Figure 1a).

% Sequence Identity of GCN5 Bromodomains					
	ScGCN5	HsGCN5L2	HsPCAF	PfGCN5	TgGCN5b
TgGCN5a	46.67	37.84	36.49	61.33	50.00
TgGCN5b	47.89	37.76	42.86	55.10	100.00

Table 1. Sequence identity of GCN5 bromodomains across select, representative species. Amino acid sequences from *S. cerevisiae* (Sc), *H. sapiens* (Hs), *P. falciparum* (Pf), and *T. gondii* (Tg). Amino acid alignment was conducted via Clustal Omega (version 1.2.4) multiple sequence alignment (shown in Figure 1a) and percentage sequence identity was determined by Clustal 2.1 analysis.

Function:

In vitro and *in vivo* studies of the GCN5 SAGA complex in yeast elucidate roles for the GCN5 bromodomain in the following areas: transcriptional activation of GCN5 co-activators [53,54], stabilization of complexes to promoter regions [55], nucleosome remodeling [54], and regulation of acetyltransferase activity [56-58].

Yeast reporter gene assays indicate that the GCN5 bromodomain is necessary for transcriptional activation of GCN5 co-activators *in vivo* [53,54]. In addition to transcriptional activation, the GCN5 bromodomain plays a role in complex/promoter association. The GCN5 bromodomain anchors the SAGA complex to acetylated promoter nucleosomes *in vitro* [55] and stabilizes the association of the Swi/Snf (Switch/Sucrose Non-Fermentable) chromatin remodeling ATPase complex within promoters *in vivo*, suggesting a role for the GCN5 bromodomain in nucleosome remodeling [54].

The GCN5 bromodomain also functions in regulating acetylation activity, including cooperativity of acetylation, and acetylation levels and specificity. In vitro nucleosome assays conducted by Li and Knaak indicate that the yeast SAGA complex acetylates H3 and H4 histone tails in nucleosomes in a cooperative fashion *in vitro*, where binding of the SAGA complex to one nucleosome increases the affinity of the complex for another, non-contiguous nucleosome [56,57]. SAGA-mediated cooperativity of nucleosome acetylation is dependent upon a functional GCN5 bromodomain [57]. Additionally, cooperative nucleosome acetylation of histone H3 tails requires that the SAGA complex

interacts with both histone H3 tails within the nucleosome, and that the histone tails exist in their proper orientation (cooperative acetylation does not occur when H3 tails are switched to H4 tail locations). These results suggest that the bromodomain may facilitate contact between non-contiguous nucleosomes through acetylated histone tail interactions [56].

Li and Knaak [57] observed that the GCN5 bromodomain increases the affinity of the SAGA complex for nucleosomes that are pre-acetylated on a single histone H3 or H4 tail. In the presence of a single pre-acetylated H3/H4 histone tail, the GCN5 bromodomain facilitates cross-tail acetylation of other histone H3 tails (without changing the site specificity of histone acetylation). Moreover, the GCN5 bromodomain increases turnover of H3 tail acetylation in the presence of a single pre-acetylated histone H4 tail within nucleosomes.

In vitro HAT assays conducted by Cieniewicz and co-workers [58] demonstrated that mutation of the GCN5 bromodomain within the GCN5/Ada2/Ada3 subcomplex in yeast decreases levels of total histone H3 acetylation, as well as alters site specificity of histone H3 acetylation in free and nucleosomal histone H3. The researchers propose a “reader/writer” model of GCN5 bromodomain regulation of specificity of acetylation, where GCN5 first acetylates histone H3 (specifically on lysine 14) and then the bromodomain anchors GCN5 to the acetylated residue to promote further acetylation activity (on H3K18).

Taken together, these studies support a model in which the bromodomain of GCN5 is necessary to anchor or stabilize GCN5 to specific regions of

chromatin to promote further GCN5 activity, such as transcriptional activation, nucleosome remodeling, and acetyltransferase activity.

1.8 Development of bromodomain inhibitors

Bromodomains have been implicated in different human disease states, including cancer, inflammation, and viral infection. As of late 2019, there were 30 clinical trials testing bromodomain inhibitors as therapeutics for human disorders [59].

There are two classes of bromodomain inhibitors: non-acetyl-lysine-competitive mimetics (e.g. ischemin) and acetyl-lysine-competitive mimetics (e.g. JQ1 and I-BET 151) [60]. The former class engages within the acetyl lysine binding pocket of the bromodomain but does not form canonical hydrogen bonding with a conserved asparagine. This class of inhibitor functions through steric inhibition of acetyl lysine binding. The latter class is the most common type developed and directly engages with the acetyl lysine binding pocket through hydrogen bonding with a conserved asparagine, thereby, competitively inhibiting binding of acetylated lysines to the bromodomain.

In addition to bromodomain inhibitors showing therapeutic promise for human disease, a number of bromodomain inhibitors have been reported to have inhibitory activity against *Toxoplasma* and other protozoan pathogens, such as *Plasmodium falciparum, Trypanosoma brucei,* and *Trypanosoma cruzi* [29,61-64].

1.9 GCN5 bromodomain-specific inhibitors

A druggability assessment reported in [65] estimates that the bromodomains of human GCN5L2 and PCAF are highly druggable. Currently, there are two available PCAF/GCN5-specific, acetyl-lysine-competitive bromodomain inhibitors, both of which were reported in 2017.

Humphreys et al. [66] identified the inhibitor GSK4027, which displays high potency, selectivity, and cellular activity against PCAF/GCN5 bromodomains. Moustakim et al. [36] also reported a PCAF/GCN5-specific inhibitor, L-Moses (also known as L-45). At the time the work of this thesis was conducted, GSK4027 was not commercially available; thus, L-Moses was the PCAF/GCN5-specific bromodomain inhibitor that was studied. L-Moses is a triazolopthalazine-based chemical inhibitor that demonstrates potency (K_D 126 nM for human PCAF BRD, K_D 550 nM for human GCN5 BRD, and K_D 280 nM for *Plasmodium* GCN5 BRD by isothermal titration calorimetry), selectivity (>4,500-fold selective over BRD4 by differential scanning fluorimetry), and cellular activity against PCAF and GCN5 bromodomains, capable of displacing their binding to histone H3.3 in cultured HEK-293 cells (by Nanobret assay). Moustakim and colleagues reported the crystal structure of the *Plasmodium* GCN5 bromodomain (PDB identification 5tpx) complexed to L-Moses, which demonstrates that L-Moses fits within the acetyl lysine binding pocket of the PfGCN5 bromodomain. Furthermore, L-Moses displays *in vitro* stability in human and mouse liver microsomes, as well as good *in vitro* solubility and permeability. Moustakim et al. found no observable cytotoxicity after 24 hours of 10 μM L-Moses treatment of

primary peripheral blood mononuclear cells. These data suggest that L-Moses may be used in *in vivo* studies.

1.10 Hypothesis and aims

GCN5b appears to be an essential gene in *Toxoplasma* that contributes to tachyzoite viability. The lysine acetyltransferase activity of GCN5b is well-characterized; nonetheless, the function of the C-terminal bromodomain is not. GCN5 bromodomains in yeast and humans have implicated roles in GCN5 function, including stabilizing GCN5 to promoters, transcriptional activation of GCN5 co-activators, and regulating GCN5 acetyltransferase activity. Additionally, two PCAF/GCN5-specific bromodomain inhibitors, GSK4027 and L-Moses, have been reported to be potent, selective, and active against PCAF/GCN5 bromodomains. This work will focus on the inhibitor L-Moses. The crystal structure of L-Moses complexed with the GCN5 bromodomain from *Plasmodium* demonstrates that L-Moses fits within the acetyl lysine binding pocket of the PfGCN5 bromodomain. **Thus, genetic and pharmacological approaches can be used to address the importance of the GCN5b bromodomain in *Toxoplasma*. We hypothesize that the GCN5b bromodomain is critical for *Toxoplasma* viability.** To address this hypothesis, we pursued two specific aims:

Aim 1: Assess the importance of the GCN5b bromodomain in *Toxoplasma gondii* viability.

Aim 2: Determine if the TgGCN5b bromodomain is a targetable element and, if so, the effects on the parasite of targeting this domain.

CHAPTER 2: MATERIALS AND METHODS

2.1 Chemicals

Shield (CheminPharma) was prepared in absolute ethanol. L-Moses dihydrochloride was purchased from Tocris (catalog no 6251) and prepared in DMSO. Chemicals were stored at -20C.

2.2 Host cell and parasite culture

Primary human foreskin fibroblast (HFF) cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM; Corning), without antibiotic/antimycotic, supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco/Invitrogen). *Toxoplasma gondii* Type I RH strain parasites were propagated in confluent monolayers of HFF cells and cultured in DMEM, without antibiotic/antimycotic, supplemented with 1% heat-inactivated FBS as previously described [67]. Host cell and parasite cultures were maintained at 37 °C with 5% CO_2 in a humidified incubator. Plasmid DNA was transfected into RHΔ*HX* parasites by electroporation and selected in 20 μM chloramphenicol; individual clones were isolated by limiting dilution in 96-well plates as previously described [67]. For assays involving Shield-based regulation, the designated parasites were cultured with vehicle (100% EtOH) or Shield (50 or 200 nM) for 48 h.

2.3 Plasmid construction

To construct an expression plasmid to generate recombinant GCN5b bromodomain (BRD) protein in *E. coli*, a region of TgGCN5b (amino acids 877-986 of TGGT1_243440 from ToxoDB) encoding the BRD (amino acids 888-985) was amplified by PCR from a previously described plasmid [6] using the following primers which include 15bp ends that are complementary to the target site in the pGEX-4T1 plasmid: sense primer (5′-TGGATCCCCGGAATTCCAGATTCCAGGTCTTCTGCAGTG) and anti-sense primer (5′- GTCGACCCGGGAATTCCAGTTGCTTCTGCTGCTG). The TgGCN5b BRD sequence was inserted into the pGEX-4T1 vector at the EcoRI site (939) using the InFusion cloning system (Takara), for expression of TgGCN5b BRD protein fused to an N-terminal GST tag. To generate a fusion protein that expresses a mutant TgGCN5b BRD, tandem alanine substitutions (Y963A/N964A/Y970A) were created using the Q5 site-directed mutagenesis kit (NEB; E0554S) with the following primers: sense primer (5′-CAAACGATTGCTTACAAATACGCGAACGAG) and anti-sense primer (5′-CTGGTGGGCGGCCTGCCGACAGTTCTTGAAC). To generate a plasmid for inducible ectopic expression of GCN5b with a mutated bromodomain (Y963A/N964A/Y970A) in *Toxoplasma*, the ptubXFLAG::CAT plasmid encoding full-length GCN5b with N-terminal destabilization (dd) and HA tags previously described in Wang et al. [6] was subjected to Q5 site-directed mutagenesis using the same Q5 primers as listed above. The wild-type and mutant

ptubXFLAG::CAT plasmids were linearized with NotI before transfection into parasites.

2.4 Purification of GCN5b bromodomain protein

GCN5b BRD wild-type and mutant pGEX-4T1 plasmids were transformed into BL21 (DE3) competent *E. coli* cells, which were grown to an OD_{600} of 0.6–0.8 and incubated with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 15 to 18 h at 16 °C to induce protein expression. IPTG-induced bacteria were centrifuged at 20,000 rpm for 25 min at 4 °C and bacterial pellets were incubated with 20 mL of lysis buffer (50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 5% glycerol (v/v), 1% Triton X-100 (v/v), 1 mM DTT, lysozyme from chicken egg white (300 µg/mL) (Sigma Aldrich; L6876), and an EDTA-free protease inhibitor cocktail tablet (Sigma Aldrich; 11873580001)) for 1 h at 4 °C, following which 250 units of benzonase nuclease (Novagen; 2894174) and 1 mM $MgCl_2$ were added and bacteria were incubated for an additional 30 min at 4 °C. The lysed bacteria were centrifuged at 20,000 rpm for 25 min at 4 °C and the soluble protein was purified and concentrated (with a 30 kDa MWCO concentrator) using the Amicon Pro Affinity Concentration Kit for GST fusion proteins (Millipore, ACK5030GS).

2.5 In vitro binding assays

In vitro binding assays were conducted to assess recombinant GST-tagged GCN5b BRD (amino acids 888-985) binding to acetylated histone H4 peptide in a similar manner as described in [68] with minor modifications. First,

biotinylated histone peptides H4 (corresponding to residues 1-21 of human histone H4) (Epigentek, R-1007) and tetra-acetylated H4 (K5/8/12/16) (corresponding to residues 1-18 of human histone H4) (Epigentek, R-1008) were linked to streptavidin magnetic beads (NEB). To link histone peptides and streptavidin beads, 625 µL of streptavidin bead slurry was washed twice in 150 mM salt solution (150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.1% Triton X-100, 10% glycerol, 1 mM DTT) using a magnetic stand, and then resuspended in 625 µL of 150 mM salt solution. 100 µL of histone peptide (100 µM) was added to the bead slurry and incubated for 15 to 18 h at 4°C. Histone peptide-linked streptavidin beads were washed three times in 150 mM salt solution to remove unbound peptide and resuspended in 625 µL of 150 mM salt solution with 0.8% sodium azide. Bead samples were stored at 4°C. Beads linked to unmodified H4 peptide, as well as streptavidin beads alone, were used as negative controls in binding/inhibition assays. 10 µL samples of histone-linked beads and control bead samples were washed once with 150 mM salt solution and resuspended in 250 µL150 mM salt solution for binding assays or 150 mM salt solution supplemented with vehicle or inhibitor for inhibitor assays. 1 µg BRD protein was added to the beads and samples were incubated for 15 to 18 h at 4°C. Beads were then washed five times with a 500 mM salt solution (500 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.1% Triton X-100, 10% glycerol, 1 mM DTT) for a total of 1 h at 4°C. Magnetic beads were boiled in 4X SDS loading buffer (40% glycerol, 240 mM Tris/HCl pH 6.8, 8% SDS, 0.04% bromophenol blue, 5% beta-mercaptoethanol) for 10 min at 95°C to elute bound bromodomain protein. Beads

were magnetically separated from the boiled samples and the supernatant was run on an SDS page gel. GST-tagged BRD protein was detected by western blot analysis.

2.6 Western blot analyses

Protein samples were run on denaturing 4–12% Tris-acetate polyacrylamide gradient gels (Invitrogen). GST-tagged fusion proteins were probed with anti-GST rabbit polyclonal antibody (1:2000; Sigma, G7781). HA-tagged fusion proteins were probed with anti-HA rat monoclonal antibody (1:2000; Roche #11867423001). The major surface antigen p30 was probed with *Toxoplasma* P30 mouse monoclonal antibody (1:1000; Genway GWB-44A329). Anti-rabbit, anti-rat, or anti-mouse antibodies conjugated with horseradish peroxidase (1:5000; GE Healthcare) were used as secondary antibodies. Blots were visualized by electrochemiluminescence using the Chemiluminescence Western Blot Substrate (Pierce) and imaged by FluorChem E machine (ProteinSimple).

2.7 Immunofluorescence assays

Localization of proteins in *Toxoplasma* was determined by immunofluorescence assay (IFA) as previously described [6]. HFFs were grown to confluence on coverslips in a 12-well plate and inoculated with freshly lysed parasites. 18 h post-infection, medium was aspirated and coverslips were fixed with 4% paraformaldehyde in PBS for 10 min, washed with PBS, and then

permeabilized with 0.2% Triton X-100 in 3% BSA-PBS for 15 min. Plates were incubated with anti-HA rat monoclonal antibody (1:2000; Roche, #11867423001) for 2 h at room temperature, washed with PBS 3 × 5 minutes, and incubated with anti-rat Alexa Fluor 488 antibody (1:2000; Invitrogen, A-11006) and 4′,6-diamidino-2-phenylindole (DAPI) (1:1000) for 1 h at room temperature. Samples were washed once more with PBS three times for 5 min each and visualized using a Nikon Eclipse 80i microscope.

2.8 GCN5 bromodomain protein alignment

Amino acid sequences utilized for protein alignment of GCN5 bromodomains are as follows: *Saccharomyces cerevisiae* (Sc) GCN5 (Uniprot entry Q03330) (aa 344-414); *Homo sapiens* (Hs) GCN5L2 (Uniprot entry Q92830) (aa 732-831); *Homo sapiens* (Hs) PCAF (Uniprot Entry Q92831) (aa 727-826); *Plasmodium falciparum* (Pf) GCN5 (PlasmoDB entry PF3D7_0823300) (aa 1352-1461); *Toxoplasma gondii* (Tg) GCN5a (ToxoDB entry TGGT1_254555-t26_1) (aa 1109-1183); *Toxoplasma gondii* (Tg) GCN5b (ToxoDB entry TGGT1_243440-t26_1) (aa 888-985). Amino acid alignment was conducted via Clustal Omega (version 1.2.4) multiple sequence alignment [69] and percentage sequence identity was determined by Clustal 2.1 analysis.

2.9 Modeling of TgGCN5b bromodomain

The predicted 3D structure of the *Toxoplasma* GCN5b BRD (amino acids 877-986) was obtained via I-Tasser using unbiased settings [70-72] and

visualized with UCSF Chimera [73]. The UCSF matchmaker function was used to superimpose BRD structures.

2.10 Parasite growth assays

For all growth assays, confluent HFF monolayers were inoculated with freshly harvested intracellular parasites that were scraped, syringe-lysed with a 25-gauge needle, and counted by hemocytometer.

Plaque assays were used to measure parasite viability [67]. 500 parasites were used to inoculate HFF monolayers in a 12-well plate. Parasite lines were added in technical triplicate within each plate. After two hours of invasion, uninvaded parasites were aspirated, wells were washed twice with medium, and fresh medium supplemented with vehicle or drug was added. Plates were left to incubate undisturbed for five to seven days and then fixed with 100% ice-cold methanol and stained with crystal violet for visualization of plaques. Plates were imaged by FluorChem E machine (ProteinSimple) and host cell lysis was calculated using ImageJ analysis [74]. Average percentages of host cell area lysed were compared between parasite lines by One-way ANOVA with Dunnett's multiple comparisons post-hoc.

The IC_{50} of L-Moses was determined by reporter assay established by McFadden et al. [7] with the modifications reported in Varberg et al. [75]. 100 RHβ1 parasites stably expressing bacterial β-galactosidase were added to each well of a black 96-well plate. After 2 h of invasion, wells were washed twice with DMEM lacking phenol red (Thermofisher Scientific), and phenol red-free DMEM

supplemented with vehicle or drug was added. Drug was serially diluted across the plate by multichannel pipette. The IC_{50} of pyrimethamine has been previously established by β-galactosidase assay and thus was used as a control [7]. After four days of incubation, 100 μM of the β-galactosidase substrate chlorophenolred-β-D-galactopyranoside (CPRG) (Sigma, #10884308001) was added to each well. 24 h later, β-galactosidase activity was measured by reading absorbance at 570 nm (excitation) and 630 nm (emission) with the Synergy H1 Plate Reader (Biotek). Assays were conducted in technical triplicate and average absorbance values obtained from three biological replicates were normalized to wells containing uninfected host cells (maximum inhibition) and infected host cells treated with vehicle (DMSO) (minimum inhibition). Absorbance values were plotted and the IC_{50} was calculated by non-linear regression-variable slope (4 parameters).

Parasite replication was measured by doubling assay [76]. Briefly, $1X10^5$ parasites were added to HFF monolayers in a 6-well plate. After 2 h of invasion, wells were washed twice with medium and fresh medium supplemented with vehicle or drug was added. Plates were fixed with 100% ice-cold methanol and stained with Hema 3 solutions I and II (Fisher, 23-122937/23-122952) at 24 and 36 h post-infection to visualize parasites and vacuoles. The number of parasites was counted in 150 random vacuoles for three biological replicates and the average percent of vacuoles containing indicated numbers of parasites between parasite lines at designated drug concentrations were compared by two-way ANOVA with Tukey's multiple comparisons post-hoc.

2.11 Host cell cytotoxicity assay

To determine the effect of L-Moses on HFF cell metabolism, a CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega; G3582) was conducted [77]. This colorimetric assay measures the reduction of the MTS tetrazolium compound to a formazan dye mediated by NAD(P)H-dependent dehydrogenase enzymes. Phenol red-free medium supplemented with vehicle (DMSO) or drug was added to confluent HFF cells in a black 96-well plate and serially diluted across the plate by multichannel pipette. Cells treated with 1% SDS were used as a positive control for inhibition of cell metabolic activity. CellTiter 96® AQueous One Solution Reagent was added to each well and incubated for four hours. The formation of formazan dye was quantified by measuring absorbance at 490 nm using the Synergy H1 Plate Reader (Biotek). Assays were performed in technical triplicate and the absorbance values from four biological replicates were averaged, the absorbance of the media alone (no cells) was subtracted, and absorbance from treated cells were normalized to absorbance from cells treated with vehicle.

2.12 Statistical analyses

All statistical analyses were conducted using GraphPad Prism version 8.3.0 for Windows, GraphPad Software, La Jolla California USA, http://www.graphpad.com.

CHAPTER 3: RESULTS

Aim 1: Assess the importance of the GCN5b bromodomain in *Toxoplasma gondii* viability

3.1 Alanine substitutions render the TgGCN5b bromodomain non-functional *in vitro*

To assess the importance of the TgGCN5b bromodomain in parasite viability, we first sought to express GCN5b with a non-functional bromodomain, characterized by an inability to recognize and bind to acetylated lysine residues. Firstly, we validated the importance of three conserved amino acids within the GCN5 bromodomain, two conserved tyrosines (Y) and an internal asparagine (N): corresponding to Y963/N964/Y970 in *Toxoplasma* GCN5b (Figure 1A). These amino acid residues line the acetyl-lysine binding pocket formed between the ZA and BC loops of the bromodomain (Figure 1B) and are critical for the recognition of acetylated lysine residues in *S. cerevisiae* [51,58,78], *H. sapiens* [50,79], and *P. falciparum* [36].

To validate the importance of Y963/N964/Y970 residues in the GCN5b bromodomain of *Toxoplasma*, we expressed a recombinant, GST-tagged wild-type (WT) and mutant (Y963A/N964A/Y970A) form of the GCN5b bromodomain (amino acids 877-986) in *E. coli* (Figure 1C) for use in an in vitro histone peptide-binding assay, similar to [68]. It has been demonstrated that the human and yeast PCAF/GCN5 bromodomains bind to acetylated lysine residues on histone

H4, including lysines 5,8,12, and 16 [reviewed in 52]. In our assay, biotinylated histone H4 peptide (tetra-acetylated at lysines 5/8/12/16) was linked to streptavidin beads and incubated with recombinant, GST-tagged bromodomain protein. Streptavidin beads alone and unacetylated histone H4 peptide were utilized as negative controls for bromodomain binding. The interaction between bromodomain protein and histone peptide was captured by probing with anti-GST antibody. WT GCN5b bromodomain protein bound to acetylated histone H4 peptide; however, the mutant form of the GCN5b bromodomain protein was unable to bind to acetylated H4 (Figure 1D).

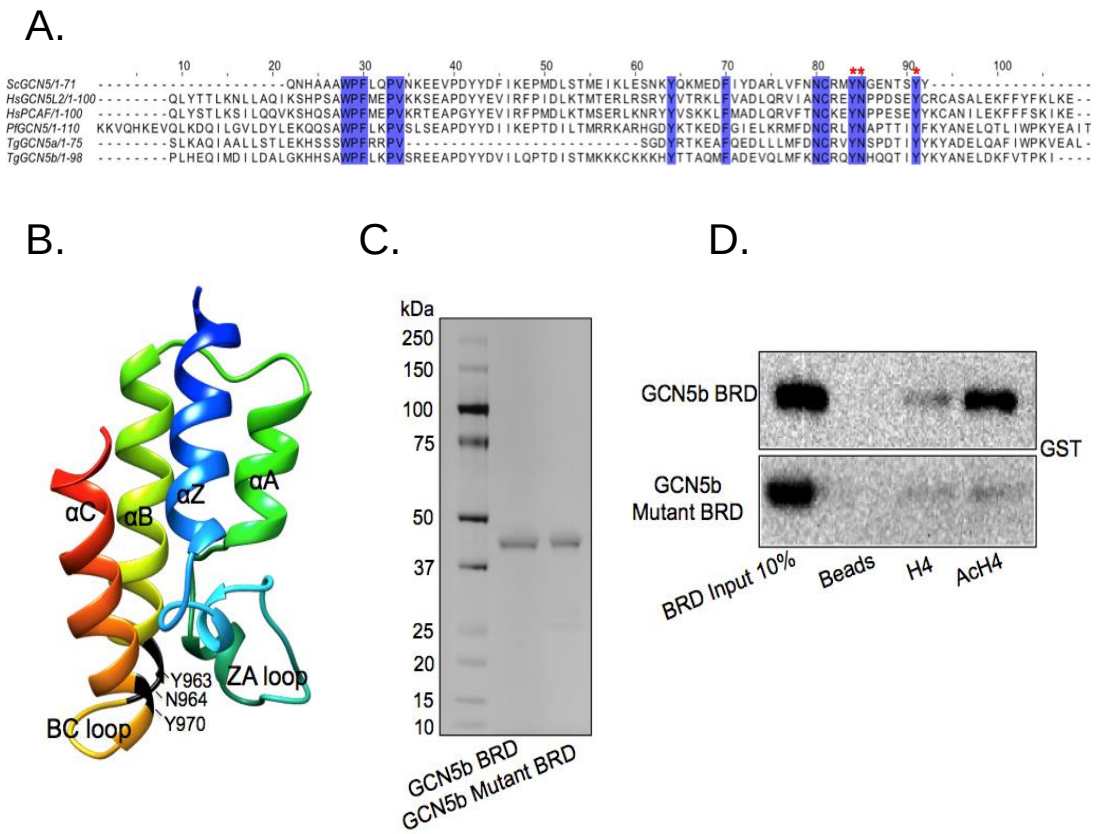
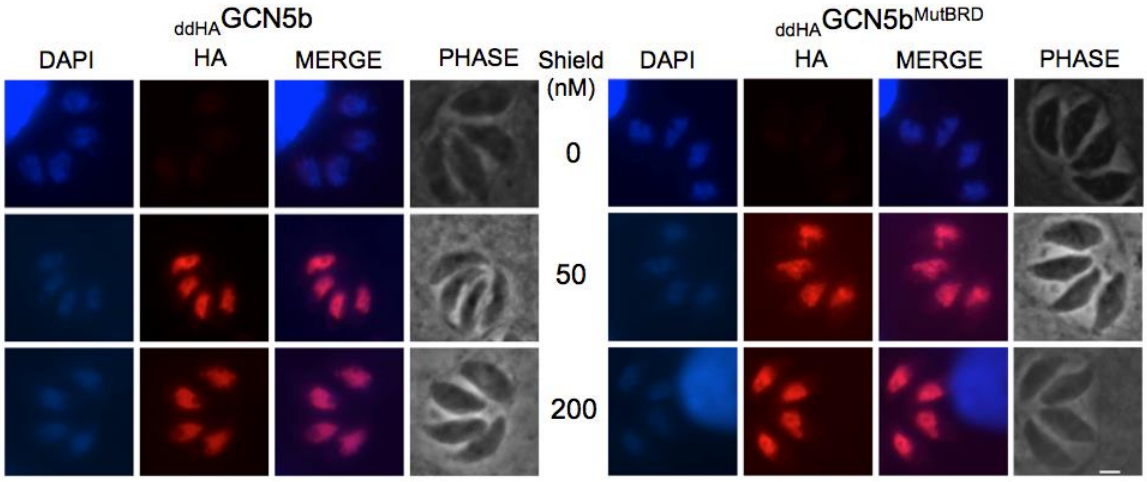


Figure 1. Alanine mutations render the TgGCN5b bromodomain non-functional *in vitro.* (A) Alignment of amino acid sequences from GCN5 bromodomains in *Saccharomyces cerevisiae* (Sc), *Homo sapiens* (Hs), *Plasmodium falciparum* (Pf), and *Toxoplasma gondii* (Tg). Highlighted amino acids are conserved among species. Red asterisks denote amino acids that were mutated to alanine in this study. (B) Predicted structure of *Toxoplasma* GCN5b bromodomain using I-Tasser. Amino acids that underwent alanine substitution are indicated. (C) Silver stain of recombinant, GST-tagged wild-type and mutant GCN5b bromodomain (BRD) proteins purified from *E. coli*. (D) BRD binding assay to monitor wild-type and mutant GCN5b BRD association with histone H4 (unmodified or acetylated, Ac), probed with anti-GST.

3.2 The bromodomain of GCN5b is important for parasite fitness

Following confirmation that the acetyl lysine binding activity of the GCN5b bromodomain can be ablated *in vitro* by alanine substitution at Y963/N964/Y970, a dominant-negative strategy, similar to that of [6], was employed. GCN5b was ectopically expressed with and without mutations at these critical residues, to determine the effect of a non-functional bromodomain on parasite viability. The premise of this approach is that the ectopic copy of GCN5b with mutations in the bromodomain will attenuate the function of the endogenous GCN5b bromodomain through competition for interactions with proteins and/or chromatin important for forming distinct GCN5 complexes, described in [44]. Using this dominant-negative approach, clonal transgenic parasites in type I RH strain that express full-length GCN5b, with or without mutations (Y963A/N964A/Y970A) in the bromodomain were generated. Both WT and mutant GCN5b were fused to an N-terminal destabilization domain (dd) and HA epitope tag, denoted as $_{ddHA}$GCN5b or $_{ddHA}$GCN5b^{MutBRD}. The dd tag sends the fusion protein to the proteasome for degradation unless it is blocked by including the ligand Shield in the culture media [80]. Expression of ectopic $_{ddHA}$GCN5b or $_{ddHA}$GCN5b^{MutBRD} was controllable by Shield, as assessed by immunofluorescence assay (IFA) and western blotting for the HA tag (Figure 2A-B). Importantly, neither the bromodomain mutations nor the dd disrupt nuclear localization of GCN5b (Figure 2B).

A.


ddHAGCN5b
DAPI
HA
MERGE
PHASE
Shield (nM)
ddHAGCN5bMutBRD
DAPI
HA
MERGE
PHASE
0
50
200

B.

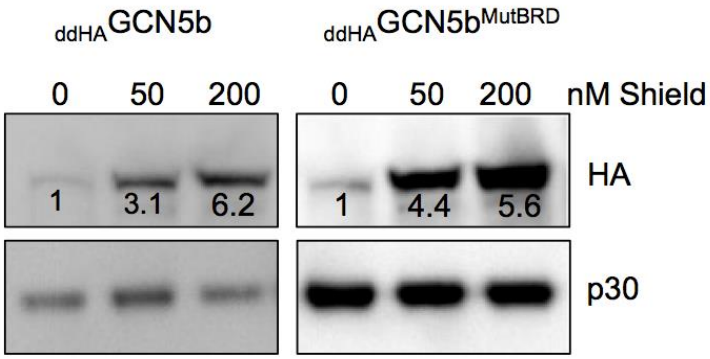

ddHAGCN5b
ddHAGCN5bMutBRD
0
50
200
0
50
200
nM Shield
1
3.1
6.2
1
4.4
5.6
HA
p30

Figure 2. Inducible expression of $_{ddHA}$GCN5b and $_{ddHA}$GCN5b^{MutBRD} RHΔ*HX* parasites via Shield regulation. (A and B) $_{ddHA}$GCN5b and $_{ddHA}$GCN5b^{MutBRD} parasites after 48 hours of incubation with vehicle or Shield. (A) Immunofluorescence assays to monitor expression of $_{ddHA}$GCN5b and $_{ddHA}$GCN5b^{MutBRD} by Shield. Blue indicates DAPI stain and red anti-HA stain. Scale bar represents 2 μm. (B) Western blot to monitor expression of $_{ddHA}$GCN5b and $_{ddHA}$GCN5b^{MutBRD} by Shield. Numeric values represent the band densities of Shield-treated parasites relative to vehicle-treated parasites, normalized to the loading control (*Toxoplasma* p30).

Standard plaque assays with the $_{ddHA}$GCN5b, $_{ddHA}$GCN5b^{MutBRD}, and parental (RHΔ*HX*) parasites were performed and parasite growth was assessed by measurement of relative area of host cell lysis. Ectopic expression of $_{ddHA}$GCN5b had no effect on parasite proliferation compared to the parental line, as found previously in [6]. Conversely, expression of $_{ddHA}$GCN5b^{MutBRD} resulted in ~35% decreased lysis of the host cell monolayer (Figure 3), suggesting that a functional GCN5b bromodomain is important for parasite viability.

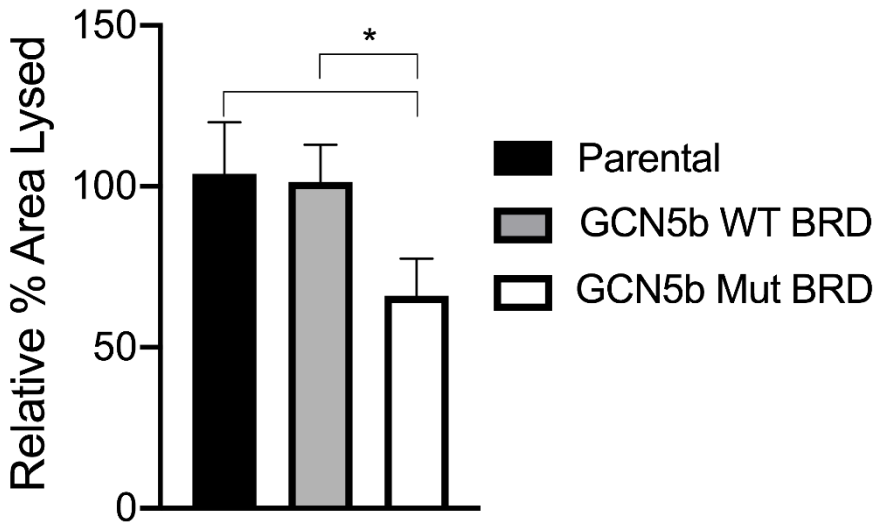


Figure 3. Ectopic expression of a mutated GCN5b bromodomain arrests parasite proliferation. Quantification of host cell area lysed in $_{ddHA}$GCN5b and $_{ddHA}$GCN5b^{MutBRD} parasites treated with 200 nM Shield relative to parental (RHΔ*HX*) parasites treated with vehicle from three independent plaque assays performed in technical triplicate. Error bars represent standard deviation. *, $P<0.05$ by One-way ANOVA with Dunnett's multiple comparisons post-hoc.

Aim 2: Determine if the TgGCN5b bromodomain is a targetable element and, if so, the effects on the parasite of targeting this domain

3.3 L-Moses inhibits GCN5b bromodomain binding activity *in vitro*

To determine if the GCN5b bromodomain in *Toxoplasma* is a targetable element, the ability of the chemical inhibitor L-Moses to interfere with GCN5b bromodomain binding to acetylated lysine residues was tested. Moustakim and colleagues [36] identified the PCAF/GCN5-specific bromodomain inhibitor L-Moses and found that it was active in HEK-293 cells against the PCAF bromodomain. Moreover, this group reported the co-crystallization structure of the GCN5 bromodomain from the apicomplexan *Plasmodium falciparum* complexed with L-Moses (PDB identification 5tpx), which illustrates that L-Moses binds within the acetyl-lysine binding pocket of the PfGCN5 bromodomain.

The program I-Tasser [70-72] was utilized to generate a predicted model of the GCN5b bromodomain from *Toxoplasma* and the superimposition of this predicted model with the co-crystallization structure of the PfGCN5 bromodomain complexed with L-Moses was generated using UCSF Chimera software [73]. The superimposed model demonstrates that the acetyl-lysine binding pocket of the TgGCN5b bromodomain shares a similar structure to that of the PfGCN5 bromodomain (Figure 4). Moreover, the amino acid residues within the *Plasmodium* GCN5 bromodomain that interact with L-Moses [36] are conserved in the *Toxoplasma* GCN5b bromodomain. Notably, two of the amino acids in the PfGCN5 bromodomain that interact with L-Moses include residues (Y1442 and

N1436, which correspond to Y970 and N964 in *Toxoplasma*, respectively) that were mutated to alanine for aim 1 of this study, further supporting the critical role of these residues in the function of the *Toxoplasma* GCN5b bromodomain.

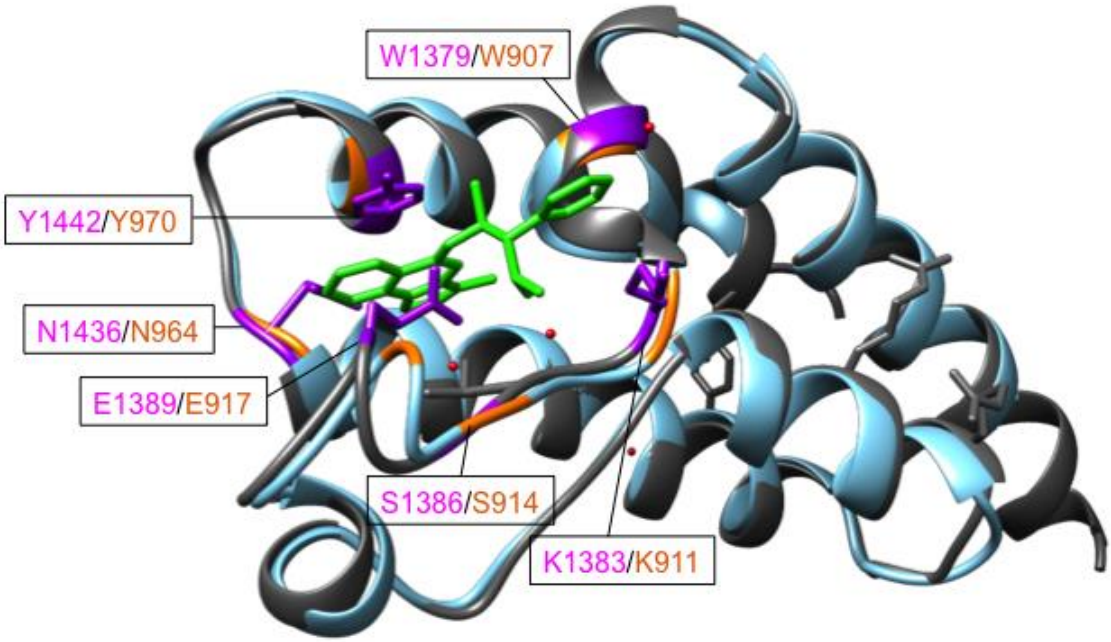


Figure 4. Modeling of PfGCN5 bromodomain complexed with L-Moses and the predicted TgGCN5b bromodomain structure. Predicted model of TgGCN5b bromodomain (blue) superimposed with the PfGCN5 bromodomain (grey) bound to L-Moses (green) (PDB identification of PfGCN5 complexed to L-Moses is 5tpx) using Chimera Matchmaker. Purple residues are those in *P. falciparum* that interact with L-Moses. Orange text represents the corresponding residues in *Toxoplasma*. Red dots represent water molecules.

Due to the similar structure and conserved critical residues between the *Plasmodium* GCN5 bromodomain and *Toxoplasma* GCN5b bromodomain, it was hypothesized that L-Moses may target the *Toxoplasma* GCN5b bromodomain. Therefore, the histone peptide binding assay and recombinant, GST-tagged WT GCN5b bromodomain protein from aim 1 were utilized to test whether L-Moses can inhibit GCN5b bromodomain binding to acetylated histone H4 K5/8/12/16 *in vitro*. Results show that L-Moses, and not the vehicle (DMSO), inhibits GCN5b bromodomain binding to acetylated histone H4 in a dose-dependent manner (Figure 5), suggesting that the activity of the *Toxoplasma* GCN5b bromodomain can be disrupted by L-Moses.

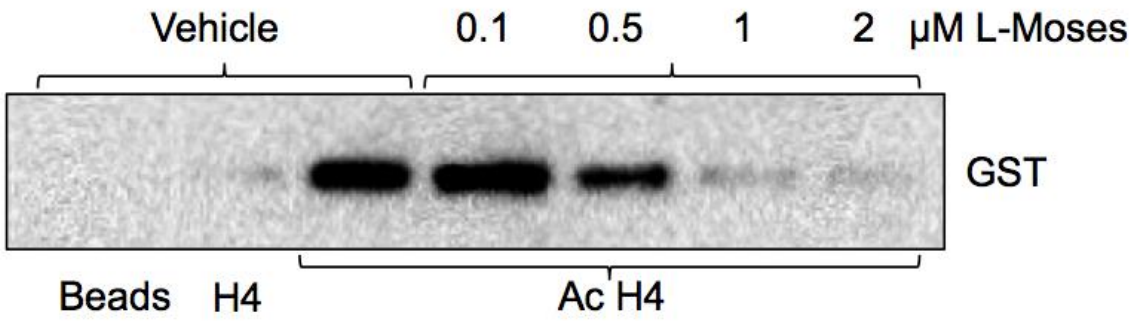


Figure 5. Treatment with L-Moses inhibits TgGCN5b bromodomain binding activity *in vitro*. BRD binding assay. Western blot was probed with anti-GST to display recombinant, GST-tagged GCN5b bromodomain binding to acetylated H4 (AcH4) in the presence of vehicle or L-Moses.

3.4. L-Moses inhibits *Toxoplasma* replication

Following confirmation that L-Moses inhibits GCN5b bromodomain binding activity *in vitro*, the effect of L-Moses on tachyzoite proliferation in HFF host cells was assessed. A reporter gene assay, established by [7], was utilized; whereby, RH strain parasites engineered to express bacterial β-galactosidase were cultured in increasing concentrations of drug, in a similar manner as [75]. Results show that L-Moses inhibits parasite proliferation at an IC_{50} of ~0.6 μM (Figure 6A). According to an MTS viability assay [77] measuring metabolic activity of HFF cells, L-Moses has >150-fold selectivity for the parasite over the host cell, as L-Moses is not cytotoxic to host cells at concentrations up to 100 μM (Figure 6B). Additionally, no overt effects on host cell morphology were observed after four days of HFF incubation with 100 μM L-Moses.

The next question addressed was whether the GCN5b bromodomain is a target of L-Moses in the parasite. It was reasoned that if GCN5b were a target, overexpression of GCN5b would confer some resistance to L-Moses. Thus, parasite growth assays using a previously generated clonal parasite line that constitutively overexpresses full-length GCN5b ($_{HA}$GCN5b), described in [81], were conducted in the presence of drug. Plaque assay indicates that L-Moses decreases viability of parasites overexpressing GCN5b to a lesser degree than parental (RHΔ*HX*) parasites, as demonstrated by smaller and fewer plaques (Figure 6C).

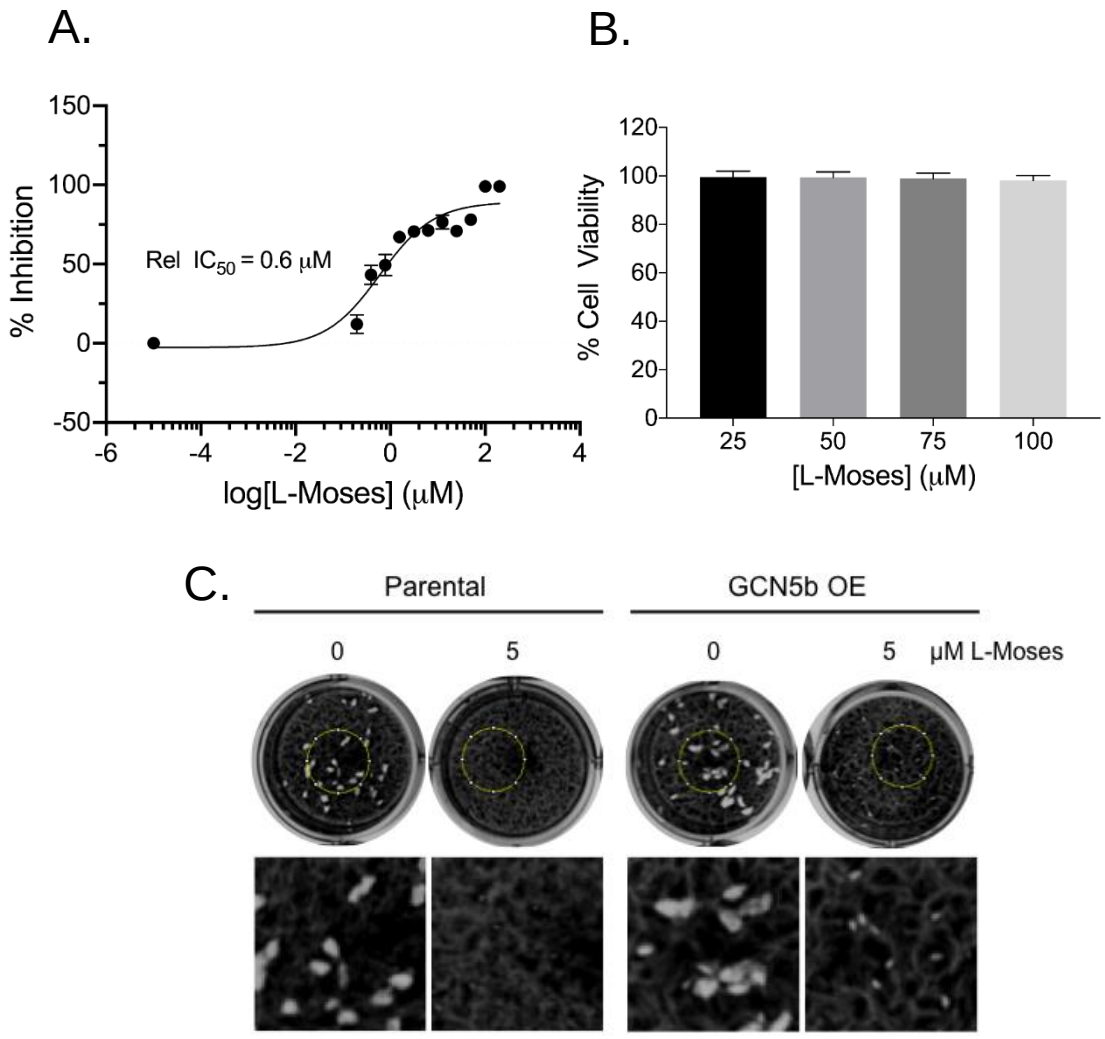

A.
B.
C.
% Inhibition
Rel IC50 = 0.6 μM
log[L-Moses] (μM)
% Cell Viability
[L-Moses] (μM)
Parental
GCN5b OE
0
5
0
5
μM L-Moses

Figure 6. Treatment with L-Moses inhibits *Toxoplasma* proliferation. (A) Proliferation assay showing average percent inhibition of the reporter activity of RH parasites constitutively expressing β-galactosidase in the presence of increasing concentrations of L-Moses. Shown are data from three independent experiments performed in technical triplicate. (B) MTS assay showing average percentage of host cell viability in L-Moses-treated cells relative to vehicle-treated cells. Data from four independent experiments performed in technical triplicate are shown. (C) Plaque assay showing fitness of parental parasites versus parasites over-expressing GCN5b, treated with vehicle or L-Moses. Images under the wells are magnified areas of the regions outlined by the yellow circle. This is representative of three independent trials showing similar results.

Furthermore, a doubling assay shows that L-Moses decreases parasite replication in the parental line compared to the GCN5b overexpression line at 36 hours post-infection (Figure 7).

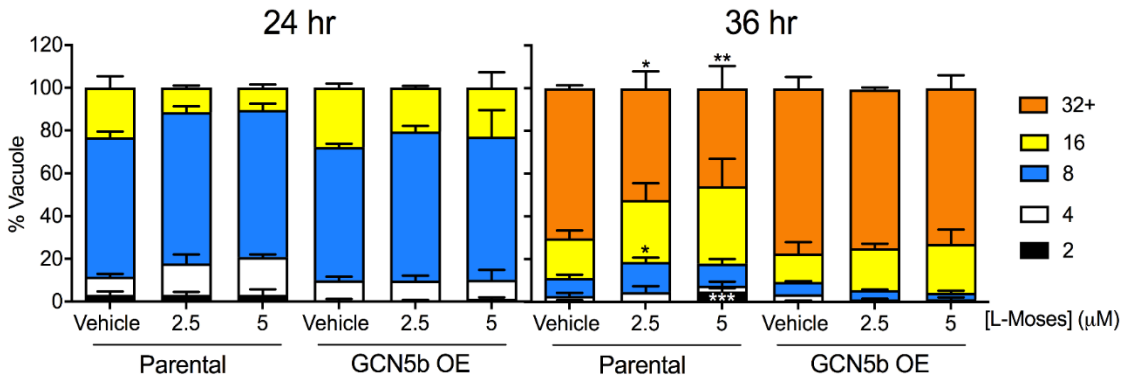


Figure 7. Treatment with L-Moses inhibits *Toxoplasma* replication. Doubling assay showing the average percent of vacuoles containing the indicated number of parasites per vacuole.150 random vacuoles were counted from three independent experiments. *, P<0.05, **, P<0.01, and ***, P<0.001 between parasite lines at the given concentration of drug by Two-way ANOVA with Tukey's multiple comparisons post-hoc. For all graphs, error bars represent standard deviation.

CHAPTER 4: DISCUSSION AND FUTURE DIRECTIONS

Aim 1: Assess the importance of the GCN5b bromodomain in *Toxoplasma gondii* viability

Results for aim 1 show that recombinant expression of GCN5b with alanine substitutions at conserved residues (Y963/N964/Y970) that line the acetyl-lysine pocket of the bromodomain ablates bromodomain binding to tetra-acetylated histone H4 peptide *in vitro*. Furthermore, ectopic expression of GCN5b with these same bromodomain mutations results in a reduction in parasite proliferation, as determined by plaque assay. These data suggest that the GCN5b bromodomain is important for parasite viability.

The molecular roles of the GCN5b bromodomain in *Toxoplasma* and GCN5b function are not known at this time. Perhaps, the TgGCN5b bromodomain plays a similar role to the reported functions of GCN5 bromodomains in yeast and humans, including roles in anchoring complexes at promoter regions [55], transcriptional activation of GCN5 co-activators [53,54], nucleosome remodeling [54], coordinating cooperative acetylation [57], and regulating acetyltransferase activity [56-58]. Additionally, the literature suggests that the GCN5b bromodomain may play a role in stabilizing GCN5b protein-protein interactions, such as those with AP2 transcription factors [6,44], through its acetyl lysine binding activity [24].

Immunoprecipitation studies followed by mass spectrometry demonstrate that GCN5 interacts with AP2 putative transcription factors in *Toxoplasma* [6,44].

Harris et al. [44] found that GCN5b is associated with at least two distinct protein complexes, characterized by interaction with different AP2 factors, during normal and alkaline pH stress conditions. Under normal pH conditions, GCN5b associates with AP2X-8 and AP2IX-7, which are subject to lysine acetylation [6], as well as AP2XII-4. Under both normal and alkaline stress conditions, GCN5b associates with ADA2-A, which is subject to lysine acetylation, as well as two PHD-finger domain-containing proteins. PHD domains have been reported as 'readers' of lysine acetylation and methylation, particularly on histone H3 [82]. These domains are the most common chromatin module with which bromodomains associate [83], such as with the tandem PHD-finger-bromodomain-containing human co-repressor KRAB-associated protein 1 (KAP1), where the PHD domain and TIF1β BRD function together as a SUMO E3 ligase to promote transcriptional gene silencing [84]. The presence of multiple acetylated proteins, as well as PHD-domain-containing proteins, in the GCN5b complex suggests that the bromodomain may play a role in stabilizing these protein interactions through its acetyl lysine binding activity. The demonstration that TgGCN5b acetylates histone H3 [8], and that yeast and human GCN5 bromodomains have been shown to interact with acetylated histone H3 residues [52], lends support to the idea that the bromodomain is important for stabilizing GCN5 KAT-mediated interactions. Additionally, Toenhake and colleagues [85] found that an AP2 factor recruits a bromodomain-containing protein to target gene promoters in *P. falciparum*. Thus, the GCN5b bromodomain may play a role in stabilizing GCN5 interaction, through its acetyl-lysine binding activity, with AP2

factors, among other proteins, to drive stage-specific gene expression in apicomplexan parasites.

Future studies to investigate the molecular functions of the GCN5b bromodomain in *Toxoplasma* could utilize the parasite lines generated in this work, as well as parasites with GCN5b bromodomain disruption introduced by a different methodology, such as endogenous mutation [5] or disruption by allelic replacement [8] or CRISPR/Cas9 technology [9,10]. However, TgGCN5b bromodomain disruption via these methods may not be possible if the bromodomain is an essential domain in GCN5b. An alternative approach could be to utilize the auxin-inducible degron (AID) system for conditional protein depletion [86]; whereby, endogenous GCN5b could be tagged with AID and then conditionally depleted by the addition of auxin. Complementation clones with various mutations in the GCN5b bromodomain could then be introduced in the presence of auxin. Studies using the parasite lines described above could assess *Toxoplasma* viability in the presence of GCN5b bromodomain disruption. Moreover, studies could investigate the role of the bromodomain in GCN5b-mediated acetyltransferase activity, gene expression, and GCN5b interaction with proteins and chromatin, as reported in [6], by western blotting, qPCR, immunoprecipitation, and chIP analyses, respectively. Studies could also investigate the role of the bromodomain in the *Toxoplasma* lytic cycle, such as in replication [76], invasion [87], and egress [88]. Understanding the roles of the GCN5b bromodomain in *Toxoplasma* will confer better understanding of the role

of the essential gene GCN5b, as well as provide insight into mechanisms and implications of targeting the bromodomain therapeutically.

Aim 2: Determine if the TgGCN5b bromodomain is a targetable element and, if so, the effects on the parasite of targeting this domain

Results for aim 2 show that the PCAF/GCN5 bromodomain inhibitor L-Moses inhibits recombinant GCN5b bromodomain binding to tetra-acetylated histone H4 peptide *in vitro.* Furthermore, L-Moses has anti-parasitic effects on tachyzoites at concentrations that do not appear to be cytotoxic to host cells. Treatment with L-Moses inhibits parasite proliferation, as evidenced by β-galactosidase assay (IC_{50} ~0.6 μM) and plaque assay. Parasites overexpressing GCN5b are less susceptible to L-Moses compared to parental parasites, as evidenced by plaque and doubling assay. These data support a model in which GCN5b is a major target of L-Moses in the parasite. Nonetheless, this work does not rule out additional mechanisms of action or targets of L-Moses.

It is worth noting that at the time this work was conducted, the negative enantiomer of L-Moses, D-45, reported in [36] was not commercially available; future studies should confirm the activity of L-Moses while using D-45 as a control.

Future studies investigating the activity of L-Moses against *Toxoplasma* GCN5b bromodomain could address: determining the precise target of L-Moses in the parasite, evaluating the selectivity of the inhibitor for the *Toxoplasma* GCN5b bromodomain, and examining the *in vivo* effects of L-Moses.

As there are 12 putative bromodomain-containing proteins in *Toxoplasma* [24], L-Moses could potentially target other *Toxoplasma* bromodomains. Preliminary work using the established bromodomain binding assay suggests that L-Moses does not inhibit recombinant GCN5a bromodomain as efficiently as GCN5b bromodomain *in vitro*. Repeat experiments could validate this finding and future studies could test the effects of L-Moses on *Toxoplasma* GCN5a. The in vitro binding assay could also be utilized to determine the effects of L-Moses on other bromodomains, as well as to identify new small molecules that interfere with the GCN5b bromodomain. Moreover, to determine the precise target of L-Moses in *Toxoplasma*, a forward genetics approach, as reported in Jeffers et al. [89], could be utilized. In this study, drug-resistant parasites were generated via chemical mutagenesis, followed by genome sequencing to identify mutations that confer drug resistance.

Furthermore, resolving a crystal structure for the GCN5b bromodomain of *Toxoplasma* complexed with L-Moses would be helpful in determining key interactions between the bromodomain and inhibitor. Interestingly, three independent reports from groups testing PCAF/GCN5-specific bromodomain inhibitors have identified a common bromodomain/inhibitor interaction: a salt bridge between a conserved glutamate residue and the inhibitor. Humphreys et al. [66] identified the PCAF/GCN5-specific bromodomain inhibitor GSK4027. They performed crystallography on the human GCN5 bromodomain bound to GSK-4027 (PDB identification 5mlj) and identified a salt-bridge interaction between E761 (corresponding to E1389 in *Plasmodium*) and the inhibitor.

Moustakim et al. [36] reported the crystal structure of L-Moses bound to the GCN5 bromodomain from *Plasmodium* and identified a salt bridge between E1389 and the dimethylamino of L-Moses. Additionally, Chu et al. [62] performed a small molecule inhibitor screen of 42 compounds predicted to target the *Plasmodium* GCN5 bromodomain through canonical participation in hydrogen bond interaction with a conserved asparagine. Of the 42 compounds screened, the most active inhibitor was the only one to have a predicted salt-bridge between the morpholine nitrogen and E1389. The reported glutamate residue (corresponding to residue 1389 in *Plasmodium* and 761 in human GCN5) involved in the salt bridge interaction with bromodomain inhibitors is also conserved in *Toxoplasma* (E917 in *Toxoplasma* GCN5b). An analysis of 26 crystal structures of human bromodomains complexed with histone tails revealed that a common interaction between bromodomains and histones is a salt bridge between the basic side chain of the histone tail and the acidic side chain of the bromodomain [90]. Ten of the 26 structures reviewed displayed the described salt bridge, and of these, the authors reported that three of the structures indicated that the salt bridge competes with the interaction between the histone tail and bromodomain, suggesting that the salt bridge interaction between the conserved glutamate residue and PCAF/GCN5-specific bromodomain inhibitors could be an interesting interaction to keep in mind when developing new inhibitors.

With regards to testing the selectivity of L-Moses for the *Toxoplasma* GCN5b bromodomain, a potential approach could mirror one described in Kumar

et al. [43]. Kumar and colleagues used comparative sequence and structural analyses to determine differences in catalytic sites of the *Plasmodium* GCN5 acetyltransferase domain compared to the human GCN5 acetyltransferase domain. Through virtual screening, this group identified known antimalarials and novel compounds with selectivity for PfGCN5 over HsGCN5. Given that the *Toxoplasma* GCN5b bromodomain shares a moderate degree of sequence identity (55%) (Table 1)(Figure 1A) and structural similarity (Figure 4) with the *Plasmodium* GCN5b bromodomain, it is likely that L-Moses will also show activity against *Plasmodium* and possibly other apicomplexan parasites.

Future studies could investigate the effects of L-Moses, or a more specific, rationally-designed GCN5b bromodomain inhibitor *in vivo*. An outstanding question to address is the effects of L-Moses against tissue cysts, as GCN5a and GCN5b appear to play roles in bradyzoite development [45,26]. *In vivo* studies could address the effects of L-Moses on tachyzoite proliferation and tissue cyst elimination in mice.

The data presented in this work suggests that the bromodomain of *Toxoplasma* GCN5b is a novel therapeutic target that is important for tachyzoite viability, and that the PCAF/GCN5 bromodomain inhibitor L-Moses is a lead compound against *Toxoplasma*.

REFERENCES

Note: Some of the information presented in this thesis, including figures, was previously published in the reference below; this information contributes to all sections of the thesis.

Hanquier, J., Gimeno, T., Jeffers, V., & Sullivan, W. J., Jr. (2020). Evaluating the GCN5b bromodomain as a novel therapeutic target against the parasite Toxoplasma gondii . Exp Parasitol, 107868. doi:10.1016/j.exppara.2020.107868

1. Kim, K., & Weiss, L. M. (2004). Toxoplasma gondii: the model apicomplexan. *International journal for parasitology, 34*(3), 423-432. doi:10.1016/j.ijpara.2003.12.009
2. Dubey, J. P., Lindsay, D. S., & Speer, C. A. (1998). Structures of Toxoplasma gondii tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts. *Clin Microbiol Rev, 11*(2), 267-299.
3. Black, M. W., & Boothroyd, J. C. (2000). Lytic cycle of Toxoplasma gondii. *Microbiol Mol Biol Rev, 64*(3), 607-623. doi:10.1128/mmbr.64.3.607-623.2000
4. Soldati, D., & Boothroyd, J. C. (1993). Transient transfection and expression in the obligate intracellular parasite Toxoplasma gondii. *Science, 260*(5106), 349-352. doi:10.1126/science.8469986

5. Huynh, M. H., & Carruthers, V. B. (2009). Tagging of endogenous genes in a Toxoplasma gondii strain lacking Ku80. *Eukaryot Cell, 8*(4), 530-539.

6. Wang, J., Dixon, S. E., Ting, L. M., Liu, T. K., Jeffers, V., Croken, M. M., . . . Sullivan, W. J., Jr. (2014). Lysine acetyltransferase GCN5b interacts with AP2 factors and is required for Toxoplasma gondii proliferation. *PLoS Pathog, 10*(1), e1003830. doi:10.1371/journal.ppat.1003830

7. McFadden, D. C., Seeber, F., & Boothroyd, J. C. (1997). Use of Toxoplasma gondii expressing beta-galactosidase for colorimetric assessment of drug activity in vitro. *Antimicrobial agents and chemotherapy, 41*(9), 1849-1853.

8. Bhatti, M. M., Livingston, M., Mullapudi, N., & Sullivan, W. J., Jr. (2006). Pair of unusual GCN5 histone acetyltransferases and ADA2 homologues in the protozoan parasite Toxoplasma gondii. *Eukaryot Cell, 5*(1), 62-76. doi:10.1128/ec.5.1.62-76.2006

9. Sidik, S. M., Huet, D., Ganesan, S. M., Huynh, M. H., Wang, T., Nasamu, A. S., . . . Lourido, S. (2016). A Genome-wide CRISPR Screen in Toxoplasma Identifies Essential Apicomplexan Genes. *Cell, 166*(6), 1423-1435 e1412.

10. Markus, B. M., Bell, G. W., Lorenzi, H. A., & Lourido, S. (2019). Optimizing Systems for Cas9 Expression in Toxoplasma gondii. *mSphere, 4*(3).

11. Gajria, B., Bahl, A., Brestelli, J., Dommer, J., Fischer, S., Gao, X., . . . Brunk, B. P. (2008). ToxoDB: an integrated Toxoplasma gondii database resource. *Nucleic Acids Res, 36*(Database issue), D553-556.

12. Hill, D. E., Chirukandoth, S., & Dubey, J. P. (2005). Biology and epidemiology of Toxoplasma gondii in man and animals. *Anim Health Res Rev, 6*(1), 41-61. doi:10.1079/ahr2005100

13. Sullivan, W. J., Jr., & Jeffers, V. (2012). Mechanisms of Toxoplasma gondii persistence and latency. *FEMS Microbiol Rev, 36*(3), 717-733. doi:10.1111/j.1574-6976.2011.00305.x

14. Basavaraju, A. (2016). Toxoplasmosis in HIV infection: An overview. Trop Parasitol, 6(2), 129-135.

15. Khurana, S., & Batra, N. (2016). Toxoplasmosis in organ transplant recipients: Evaluation, implication, and prevention. *Trop Parasitol, 6*(2), 123-128. doi:10.4103/2229-5070.190814

16. Ferguson, D. J., & Hutchison, W. M. (1987). An ultrastructural study of the early development and tissue cyst formation of Toxoplasma gondii in the brains of mice. Parasitol Res, 73(6), 483-491. doi:10.1007/bf00535321

17. Luft, B. J., & Chua, A. (2000). Central Nervous System Toxoplasmosis in HIV Pathogenesis, Diagnosis, and Therapy. Curr Infect Dis Rep, 2(4), 358-362. doi:10.1007/s11908-000-0016-x

18. Kravetz, J. (2013). Congenital toxoplasmosis. BMJ Clin Evid, 2013.

19. Alday, P. H., & Doggett, J. S. (2017). Drugs in development for toxoplasmosis: advances, challenges, and current status. *Drug Des Devel Ther, 11*, 273-293. doi:10.2147/dddt.s60973

20. Ben-Harari, R. R., Goodwin, E., & Casoy, J. (2017). Adverse Event Profile of Pyrimethamine-Based Therapy in Toxoplasmosis: A Systematic Review. *Drugs R D, 17*(4), 523-544. doi:10.1007/s40268-017-0206-8

21. Marmorstein, R., & Zhou, M. M. (2014). Writers and readers of histone acetylation: structure, mechanism, and inhibition. *Cold Spring Harb Perspect Biol, 6*(7), a018762.

22. Allis, C. D., Berger, S. L., Cote, J., Dent, S., Jenuwien, T., Kouzarides, T., . . . Zhang, Y. (2007). New nomenclature for chromatin-modifying enzymes. In Cell (Vol. 131, pp. 633-636). United States.

23. Kouzarides, T. (2000). Acetylation: a regulatory modification to rival phosphorylation? *EMBO J, 19*(6), 1176-1179.

24. Jeffers, V., & Sullivan, W. J., Jr. (2012). Lysine acetylation is widespread on proteins of diverse function and localization in the protozoan parasite Toxoplasma gondii. *Eukaryot Cell, 11*(6), 735-742. doi:10.1128/ec.00088-12

25. Miao, J., Lawrence, M., Jeffers, V., Zhao, F., Parker, D., Ge, Y., . . . Cui, L. (2013). Extensive lysine acetylation occurs in evolutionarily conserved metabolic pathways and parasite-specific functions during Plasmodium falciparum intraerythrocytic development. *Mol Microbiol, 89*(4), 660-675. doi:10.1111/mmi.12303

26. Saksouk, N., Bhatti, M. M., Kieffer, S., Smith, A. T., Musset, K., Garin, J., . . . Hakimi, M. A. (2005). Histone-modifying complexes regulate gene expression pertinent to the differentiation of the protozoan parasite

Toxoplasma gondii. *Mol Cell Biol, 25*(23), 10301-10314. doi:10.1128/mcb.25.23.10301-10314.2005

27. Nagy, Z., & Tora, L. (2007). Distinct GCN5/PCAF-containing complexes function as co-activators and are involved in transcription factor and global histone acetylation. *Oncogene, 26*(37), 5341-5357. doi:10.1038/sj.onc.1210604

28. Wang, T., Xing, J., Liu, Z., Zheng, M., Yao, Y., Hu, Z., . . . Ni, Z. (2019). Histone acetyltransferase GCN5-mediated regulation of long non-coding RNA At4 contributes to phosphate starvation response in Arabidopsis. *J Exp Bot, 70*(21), 6337-6348. doi:10.1093/jxb/erz359

29. Jeffers, V., Yang, C., Huang, S., & Sullivan, W. J., Jr. (2017). Bromodomains in Protozoan Parasites: Evolution, Function, and Opportunities for Drug Development. *Microbiol Mol Biol Rev, 81*(1).

30. Wang, L., Mizzen, C., Ying, C., Candau, R., Barlev, N., Brownell, J., . . . Berger, S. L. (1997). Histone acetyltransferase activity is conserved between yeast and human GCN5 and is required for complementation of growth and transcriptional activation. *Mol Cell Biol, 17*(1), 519-527.

31. Kuo, M. H., Zhou, J., Jambeck, P., Churchill, M. E., & Allis, C. D. (1998). Histone acetyltransferase activity of yeast Gcn5p is required for the activation of target genes in vivo. *Genes Dev, 12*(5), 627-639. doi:10.1101/gad.12.5.627

32. Xue-Franzen, Y., Johnsson, A., Brodin, D., Henriksson, J., Burglin, T. R., & Wright, A. P. (2010). Genome-wide characterisation of the Gcn5 histone

acetyltransferase in budding yeast during stress adaptation reveals evolutionarily conserved and diverged roles. *BMC Genomics, 11*, 200. doi:10.1186/1471-2164-11-200

33. Choudhary, C., Kumar, C., Gnad, F., Nielsen, M. L., Rehman, M., Walther, T. C., . . . Mann, M. (2009). Lysine acetylation targets protein complexes and co-regulates major cellular functions. In *Science* (Vol. 325, pp. 834-840). United States.

34. Yamauchi, T., Yamauchi, J., Kuwata, T., Tamura, T., Yamashita, T., Bae, N., . . . Nakatani, Y. (2000). Distinct but overlapping roles of histone acetylase PCAF and of the closely related PCAF-B/GCN5 in mouse embryogenesis. *Proc Natl Acad Sci U S A, 97*(21), 11303-11306. doi:10.1073/pnas.97.21.11303

35. Xu, W., Edmondson, D. G., Evrard, Y. A., Wakamiya, M., Behringer, R. R., & Roth, S. Y. (2000). Loss of Gcn5l2 leads to increased apoptosis and mesodermal defects during mouse development. *Nat Genet, 26*(2), 229-232. doi:10.1038/79973

36. Moustakim, M., Clark, P. G., Trulli, L., Fuentes de Arriba, A. L., Ehebauer, M. T., Chaikuad, A., . . . Brennan, P. E. (2017). Discovery of a PCAF Bromodomain Chemical Probe. *Angew Chem Int Ed Engl, 56*(3), 827-831. doi:10.1002/anie.201610816

37. Helmlinger, D., & Tora, L. (2017). Sharing the SAGA. *Trends Biochem Sci, 42*(11), 850-861. doi:10.1016/j.tibs.2017.09.001

38. Fan, Q., An, L., & Cui, L. (2004). Plasmodium falciparum histone acetyltransferase, a yeast GCN5 homologue involved in chromatin remodeling. *Eukaryot Cell, 3*(2), 264-276.

39. Rawat, M., Kanyal, A., Sahasrabudhe, A., Vembar, S. S., Lopez-Rubio, J.-J., & Karmodiya, K. (2019). PfGCN5, a global regulator of stress responsive genes, modulates artemisinin resistance in Plasmodium falciparum. *bioRxiv*, 679100. doi:10.1101/679100

40. Cui, L., Miao, J., Furuya, T., Li, X., & Su, X. Z. (2007). PfGCN5-mediated histone H3 acetylation plays a key role in gene expression in Plasmodium falciparum. *Eukaryot Cell, 6*(7), 1219-1227. doi:10.1128/ec.00062-07

41. Cui, L., & Miao, J. (2007). Cytotoxic effect of curcumin on malaria parasite Plasmodium falciparum: inhibition of histone acetylation and generation of reactive oxygen species. *Antimicrob Agents Chemother, 51*(2), 488-494. doi:10.1128/aac.01238-06

42. Srivastava, S., Bhowmick, K., Chatterjee, S., Basha, J., Kundu, T. K., & Dhar, S. K. (2014). Histone H3K9 acetylation level modulates gene expression and may affect parasite growth in human malaria parasite Plasmodium falciparum. *Febs j, 281*(23), 5265-5278. doi:10.1111/febs.13067

43. Kumar, A., Bhowmick, K., Vikramdeo, K. S., Mondal, N., Subbarao, N., & Dhar, S. K. (2017). Designing novel inhibitors against histone acetyltransferase (HAT: GCN5) of Plasmodium falciparum. *Eur J Med Chem, 138*, 26-37. doi:10.1016/j.ejmech.2017.06.009

44. Harris, M. T., Jeffers, V., Martynowicz, J., True, J. D., Mosley, A. L., & Sullivan, W. J., Jr. (2019). A novel GCN5b lysine acetyltransferase complex associates with distinct transcription factors in the protozoan parasite Toxoplasma gondii. *Mol Biochem Parasitol, 232*, 111203. doi:10.1016/j.molbiopara.2019.111203

45. Naguleswaran, A., Elias, E. V., McClintick, J., Edenberg, H. J., & Sullivan, W. J., Jr. (2010). Toxoplasma gondii lysine acetyltransferase GCN5-A functions in the cellular response to alkaline stress and expression of cyst genes. *PLoS Pathog, 6*(12), e1001232. doi:10.1371/journal.ppat.1001232

46. Jeffers, V., Gao, H., Checkley, L. A., Liu, Y., Ferdig, M. T., & Sullivan, W. J., Jr. (2016). Garcinol Inhibits GCN5-Mediated Lysine Acetyltransferase Activity and Prevents Replication of the Parasite Toxoplasma gondii. *Antimicrob Agents Chemother, 60*(4), 2164-2170. doi:10.1128/aac.03059-15

47. Tamkun, J. W., Deuring, R., Scott, M. P., Kissinger, M., Pattatucci, A. M., Kaufman, T. C., & Kennison, J. A. (1992). brahma: a regulator of Drosophila homeotic genes structurally related to the yeast transcriptional activator SNF2/SWI2. *Cell, 68*(3), 561-572. doi:10.1016/0092-8674(92)90191-e

48. Filippakopoulos, P., Picaud, S., Mangos, M., Keates, T., Lambert, J.-P., Barsyte-Lovejoy, D., . . . Knapp, S. (2012). Histone recognition and large-scale structural analysis of the human bromodomain family. *Cell, 149*(1), 214-231. doi:10.1016/j.cell.2012.02.013

49. Zhang, Q., Chakravarty, S., Ghersi, D., Zeng, L., Plotnikov, A. N., Sanchez, R., & Zhou, M. M. (2010). Biochemical profiling of histone binding selectivity of the yeast bromodomain family. *PLoS One, 5*(1), e8903. doi:10.1371/journal.pone.0008903

50. Dhalluin, C., Carlson, J. E., Zeng, L., He, C., Aggarwal, A. K., & Zhou, M. M. (1999). Structure and ligand of a histone acetyltransferase bromodomain. *Nature, 399*(6735), 491-496. doi:10.1038/20974

51. Owen, D. J., Ornaghi, P., Yang, J. C., Lowe, N., Evans, P. R., Ballario, P., . . . Travers, A. A. (2000). The structural basis for the recognition of acetylated histone H4 by the bromodomain of histone acetyltransferase gcn5p. *Embo j, 19*(22), 6141-6149. doi:10.1093/emboj/19.22.6141

52. Filippakopoulos, P., & Knapp, S. (2012). The bromodomain interaction module. *FEBS Lett, 586*(17), 2692-2704.doi:10.1016/j.febslet.2012.04.045

53. Georgakopoulos, T., Gounalaki, N., & Thireos, G. (1995). Genetic evidence for the interaction of the yeast transcriptional co-activator proteins GCN5 and ADA2. *Mol Gen Genet, 246*(6), 723-728. doi:10.1007/bf00290718

54. Syntichaki, P., Topalidou, I., & Thireos, G. (2000). The Gcn5 bromodomain co-ordinates nucleosome remodelling. *Nature, 404*(6776), 414-417. doi:10.1038/35006136

55. Hassan, A. H., Prochasson, P., Neely, K. E., Galasinski, S. C., Chandy, M., Carrozza, M. J., & Workman, J. L. (2002). Function and selectivity of bromodomains in anchoring chromatin-modifying complexes to promoter nucleosomes. In *Cell* (Vol. 111, pp. 369-379). United States.

56. Li, S., & Shogren-Knaak, M. A. (2008). Cross-talk between histone H3 tails produces cooperative nucleosome acetylation. *Proc Natl Acad Sci U S A, 105*(47), 18243-18248. doi:10.1073/pnas.0804530105

57. Li, S., & Shogren-Knaak, M. A. (2009). The Gcn5 bromodomain of the SAGA complex facilitates cooperative and cross-tail acetylation of nucleosomes. *J Biol Chem, 284*(14), 9411-9417. doi:10.1074/jbc.M809617200

58. Cieniewicz, A. M., Moreland, L., Ringel, A. E., Mackintosh, S. G., Raman, A., Gilbert, T. M., . . . Taverna, S. D. (2014). The bromodomain of Gcn5 regulates site specificity of lysine acetylation on histone H3. *Mol Cell Proteomics, 13*(11), 2896-2910. doi:10.1074/mcp.M114.038174

59. Zaware, N., & Zhou, M. M. (2019). Bromodomain biology and drug discovery. *Nat Struct Mol Biol, 26*(10), 870-879. doi:10.1038/s41594-019-0309-8

60. Filippakopoulos, P., & Knapp, S. (2014). Targeting bromodomains: epigenetic readers of lysine acetylation. *Nat Rev Drug Discov, 13*(5), 337-356. doi:10.1038/nrd4286

61. Garcia, P., Alonso, V. L., Serra, E., Escalante, A. M., & Furlan, R. L. E. (2018). Discovery of a Biologically Active Bromodomain Inhibitor by Target-Directed Dynamic Combinatorial Chemistry. *ACS Med Chem Lett, 9*(10), 1002-1006. doi:10.1021/acsmedchemlett.8b00247

62. Chua, M. J., Robaa, D., Skinner-Adams, T. S., Sippl, W., & Andrews, K. T. (2018). Activity of bromodomain protein inhibitors/binders against asexual-stage Plasmodium falciparum parasites. *Int J Parasitol Drugs Drug Resist, 8*(2), 189-193. doi:10.1016/j.ijpddr.2018.03.001

63. Schulz, D., Mugnier, M. R., Paulsen, E. M., Kim, H. S., Chung, C. W., Tough, D. F., . . . Debler, E. W. (2015). Bromodomain Proteins Contribute to Maintenance of Bloodstream Form Stage Identity in the African Trypanosome. *PLoS Biol, 13*(12), e1002316. doi:10.1371/journal.pbio.1002316

64. Ramallo, I. A., Alonso, V. L., Rua, F., Serra, E., & Furlan, R. L. E. (2018). A Bioactive Trypanosoma cruzi Bromodomain Inhibitor from Chemically Engineered Extracts. *ACS Comb Sci, 20*(4), 220-228. doi:10.1021/acscombsci.7b00172

65. Vidler, L. R., Brown, N., Knapp, S., & Hoelder, S. (2012). Druggability analysis and structural classification of bromodomain acetyl-lysine binding sites. *J Med Chem, 55*(17), 7346-7359. doi:10.1021/jm300346w

66. Humphreys, P. G., Bamborough, P., Chung, C. W., Craggs, P. D., Gordon, L., Grandi, P., . . . Prinjha, R. K. (2017). Discovery of a Potent, Cell Penetrant, and Selective p300/CBP-Associated Factor (PCAF)/General Control Nonderepressible 5 (GCN5) Bromodomain Chemical Probe. J Med Chem, 60(2), 695-709. doi:10.1021/acs.jmedchem.6b01566

67. Roos, D. S., Donald, R. G., Morrissette, N. S., & Moulton, A. L. (1994). Molecular tools for genetic dissection of the protozoan parasite Toxoplasma gondii. *Methods Cell Biol, 45*, 27-63. doi:10.1016/s0091-679x(08)61845-2

68. Xu, L., Cheng, A., Huang, M., Zhang, J., Jiang, Y., Wang, C., . . . Ruan, K. (2017). Structural insight into the recognition of acetylated histone

H3K56ac mediated by the bromodomain of CREB-binding protein. *Febs j, 284*(20), 3422-3436. doi:10.1111/febs.14198

69. Sievers, F., & Higgins, D. G. (2018). Clustal Omega for making accurate alignments of many protein sequences. *Protein Sci, 27*(1), 135-145. doi:10.1002/pro.3290

70. Zhang, Y. (2008). I-TASSER server for protein 3D structure prediction. *BMC Bioinformatics, 9*, 40. doi:10.1186/1471-2105-9-40

71. Roy, A., Kucukural, A., & Zhang, Y. (2010). I-TASSER: a unified platform for automated protein structure and function prediction. *Nat Protoc, 5*(4), 725-738. doi:10.1038/nprot.2010.5

72. Yang, J., Yan, R., Roy, A., Xu, D., Poisson, J., & Zhang, Y. (2015). The I-TASSER Suite: protein structure and function prediction. In *Nat Methods* (Vol. 12, pp. 7-8). United States.

73. Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., & Ferrin, T. E. (2004). UCSF Chimera--a visualization system for exploratory research and analysis. *J Comput Chem, 25*(13), 1605-1612. doi:10.1002/jcc.20084

74. Rueden, C. T., Schindelin, J., Hiner, M. C., DeZonia, B. E., Walter, A. E., Arena, E. T., & Eliceiri, K. W. (2017). ImageJ2: ImageJ for the next generation of scientific image data. *BMC Bioinformatics, 18*(1), 529. doi:10.1186/s12859-017-1934-z

75. Varberg, J. M., LaFavers, K. A., Arrizabalaga, G., & Sullivan, W. J., Jr. (2018). Characterization of Plasmodium Atg3-Atg8 Interaction Inhibitors

Identifies Novel Alternative Mechanisms of Action in Toxoplasma gondii. *Antimicrob Agents Chemother, 62*(2). doi:10.1128/aac.01489-17

76. Fichera, M. E., Bhopale, M. K., & Roos, D. S. (1995). In vitro assays elucidate peculiar kinetics of clindamycin action against Toxoplasma gondii. *Antimicrob Agents Chemother, 39*(7), 1530-1537. doi:10.1128/aac.39.7.1530
77. Doggett, J. S., Nilsen, A., Forquer, I., Wegmann, K. W., Jones-Brando, L., Yolken, R. H., . . . Riscoe, M. K. (2012). Endochin-like quinolones are highly efficacious against acute and latent experimental toxoplasmosis. *Proc Natl Acad Sci U S A, 109*(39), 15936-15941. doi:10.1073/pnas.1208069109
78. Hudson, B. P., Martinez-Yamout, M. A., Dyson, H. J., & Wright, P. E. (2000). Solution structure and acetyl-lysine binding activity of the GCN5 bromodomain. *J Mol Biol, 304*(3), 355-370. doi:10.1006/jmbi.2000.4207
79. Chaikuad, A., Lang, S., Brennan, P. E., Temperini, C., Fedorov, O., Hollander, J., . . . Knapp, S. (2016). Structure-Based Identification of Inhibitory Fragments Targeting the p300/CBP-Associated Factor Bromodomain. *J Med Chem, 59*(4), 1648-1653.
80. Herm-Gotz, A., Agop-Nersesian, C., Munter, S., Grimley, J. S., Wandless, T. J., Frischknecht, F., & Meissner, M. (2007). Rapid control of protein level in the apicomplexan Toxoplasma gondii. *Nat Methods, 4*(12), 1003-1005. doi:10.1038/nmeth1134

81. Bhatti, M. M., & Sullivan, W. J., Jr. (2005). Histone acetylase GCN5 enters the nucleus via importin-alpha in protozoan parasite Toxoplasma gondii. *J Biol Chem, 280*(7), 5902-5908. doi:10.1074/jbc.M410656200

82. Sanchez, R., & Zhou, M. M. (2011). The PHD finger: a versatile epigenome reader. *Trends Biochem Sci, 36*(7), 364-372. doi:10.1016/j.tibs.2011.03.005

83. Sanchez, R., & Zhou, M. M. (2009). The role of human bromodomains in chromatin biology and gene transcription. *Curr Opin Drug Discov Devel, 12*(5), 659-665.

84. Zeng, L., Yap, K. L., Ivanov, A. V., Wang, X., Mujtaba, S., Plotnikova, O., . . . Zhou, M. M. (2008). Structural insights into human KAP1 PHD finger-bromodomain and its role in gene silencing. *Nat Struct Mol Biol, 15*(6), 626-633.

85. Toenhake, C. G., Fraschka, S. A., Vijayabaskar, M. S., Westhead, D. R., van Heeringen, S. J., & Bartfai, R. (2018). Chromatin Accessibility-Based Characterization of the Gene Regulatory Network Underlying Plasmodium falciparum Blood-Stage Development. *Cell Host Microbe, 23*(4), 557-569 e559.

86. Brown, K. M., Long, S., & Sibley, L. D. (2018). Conditional Knockdown of Proteins Using Auxin-inducible Degron (AID) Fusions in Toxoplasma gondii. *Bio Protoc, 8*(4). doi:10.21769/BioProtoc.2728

87. Kafsack, B. F., Beckers, C., & Carruthers, V. B. (2004). Synchronous invasion of host cells by Toxoplasma gondii. In *Mol Biochem Parasitol* (Vol. 136, pp. 309-311). Netherlands.

88. Lourido, S., Tang, K., & Sibley, L. D. (2012). Distinct signalling pathways control Toxoplasma egress and host-cell invasion. *Embo j, 31*(24), 4524-4534. doi:10.1038/emboj.2012.299

89. Jeffers, V., Kamau, E. T., Srinivasan, A. R., Harper, J., Sankaran, P., Post, S. E., . . . Boyle, J. P. (2017). TgPRELID, a Mitochondrial Protein Linked to Multidrug Resistance in the Parasite Toxoplasma gondii. *mSphere, 2*(1). doi:10.1128/mSphere.00229-16

90. Marchand, J. R., & Caflisch, A. (2015). Binding Mode of Acetylated Histones to Bromodomains: Variations on a Common Motif. ChemMedChem, 10(8), 1327-1333. doi:10.1002/cmdc.201500141

CURRICULUM VITAE

Jocelyne Nicole Hanquier

EDUCATION:

Master of Science in Microbiology and Immunology (2020)

Indiana University at IUPUI, Indianapolis, IN

Bachelor of Science in Biology, *Summa cum laude* (2017)

Marian University, Indianapolis, IN

PUBLICATIONS:

Hanquier, J., Gimeno, T., Jeffers, V., & Sullivan, W. J. (2020). Evaluating the GCN5b bromodomain as a novel therapeutic target against the parasite *Toxoplasma gondii*. *Experimental Parasitology*, 107868. doi: 10.1016/j.exppara.2020.107868

RESEARCH PRESENTATIONS:

GCN5 bromodomains in Toxoplasma gondii: Investigating their function and potential as drug targets, **Jocelyne Hanquier**, Thomas Gimeno, Dr. Victoria Jeffers, Dr. William J. Sullivan. Biology of Intracellular Pathogens Retreat, Indiana University School of Medicine, Indianapolis, IN. June 2018. **Poster**.

STUDENT MENTORING AT THE IU SCHOOL OF MEDICINE:

• Thomas Gimeno-Project STEM student (Summer 2018 and Summer 2019)

HONORS AND AWARDS:

• Michael J. Eoff Award for Research Excellence at Marian University (Fall 2015 and Spring 2016)

• Thoreau Award of Excellence in Science Writing at Marian University (Fall 2013)

AFFILIATIONS:

• Sigma Zeta National Science and Mathematics Honor Society at Marian University (2015-2017)

• Indiana Academy of Science (2015-2016)

INTERNSHIP EXPERIENCE:

• Eli Lilly, Tissue Sample Management and Acquisition, LRL Operations, Summer Replacement Student (2015)